Platelet Apoptosis in Health and Disease

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Universität Zürich

von

Jeannine Winkler

von

Winterthur, ZH

Promotionskomitee

Prof. Dr. Michael Hengartner (Vorsitz)

Dr. Oliver Speer (Leitung der Dissertation)

PD Dr. med. Markus Schmugge

Prof. Dr. Margaret Rand

Prof. Dr. med. Christoph Renner

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Zusammenfassung

Primäre Immun-Thrombozytopenie (ITP) ist eine verbreitete und benigne Autoimmunkrankheit, welche die Thrombozyten betrifft. Pädiatrische ITP kommt häufig nach viralen Infekten vor und wird vermutlich durch eine Produktion von Autoantikörpern gegen Glykoproteine der Thrombozytenoberfläche verursacht. Vieles weist darauf hin, dass diese Autoantikörper am beschleunigten Abbau der Thrombozyten beteiligt sein könnten, welcher in ITP Patienten festgestellt wird und zu einer tiefen Thrombozytenzahl führt. Charakteristisch für die ITP sind Spontanblutungen, wobei es sich meistens um Petechien handelt; punktförmige Haut- und Schleimhautblutungen. Pro Jahr treten etwa 100 Neuerkrankungen in einer Million Menschen auf, wobei die Hälfte der betroffenen Patienten Kinder sind. Die meisten Fälle der akuten ITP heilen spontan oder nach Therapie innerhalb von 3 Monaten aus. Jedoch besteht das Risiko für möglicherweise fatale Hirnblutungen, die bei etwa 0.1 – 0.2% der Patienten auftreten. Zudem manifestiert sich die Krankheit in ungefähr 10% der Patienten länger als 1 Jahr und entwickelt sich zu einer chronischen ITP. ITP Patienten werden mit intravenösen Immunglobulinen (IVIg) behandelt, welche einen Anstieg in der Thrombozytenzahl bewirken. Die derzeitige Hypothese zum Wirkmechanismus der IVIg nimmt an, dass IVIg die Fc Rezeptoren der Makrophagen blockiert und dadurch den erhöhten Abbau der Thrombozyten verhindert. Dieser Hypothese entsprechend führte die Verabreichung von anti-Thrombozyten Antikörpern in Mäusen zu einer schweren Thrombozytopenie. Jedoch wurden in diesen Thrombozyten apoptotische Vorgänge beobachtet. Sowohl Thrombozytopenie wie auch die apoptotischen Vorgänge in den Thrombozyten wurden im Mausmodell durch IVIg verbessert. Bis jetzt war nicht bekannt, ob diese apoptotischen Vorgänge auch in der pädiatrischen ITP vorkommen.

Programmierter Zelltod, auch Apoptose genannt, ist der Hauptmechanismus, der die zelluläre Lebensspanne reguliert, aber auch zur Eliminierung von beschädigten nukleären Zellen beiträgt. Entscheidend in der apoptotischen Signalkaskade sind Caspasen, welche eine proteolytische Funktion haben. Caspase-8 ist Teil der extrinsischen, Caspase-9 Teil der intrinsischen apoptotischen Signalkaskade. Aktivierung von Caspase-8 oder -9 führt zu aktiver Caspase-3 und -7. Diese Effektor-Caspasen schneiden spezifische Substrate, was zum Tod der Zelle führt. Aktive Caspase-9, -3 und -7 werden durch XIAP, ein X-chromosomales anti-apoptotisches Protein, inhibiert. XIAP wiederum wird von der pro-apoptotischen Protease Omi/HtrA2 inaktiviert. Omi/HtrA2 ist eine Komponente der intrinsischen Signalkaskade und wird nach Permeabilisierung der äusseren mitochondrialen Membran von Mitochondrien freigesetzt. Auch in den anukleären Thrombozyten wurden apoptotische Vorgänge festgestellt, unter anderem die mitochondriale Freisetzung von

Cytochrom c, eine Aktivierung von Caspase-3 oder ein Anstieg von Mikropartikel. Das Vorkommen von XIAP wie auch Omi/HtrA2 in Thrombozyten wurde jedoch noch nicht beschrieben. Auch in anderen Erkrankungen wie zum Beispiel beim Bernhard Soulier Syndrom oder beim Diabetes Mellitus Typ 2 wurden apoptotische Vorgänge im Thrombozyten beobachtet. Gleichzeitig kommen in diesen Krankheiten oft auch Thrombozytendefekte vor. Thrombozytenapoptose und Thrombozytenfunktion wurden noch nicht in einer pädiatrischen ITP Population vor und nach IVIg Behandlung untersucht. Ausserdem wurden in Thrombozyten noch nicht alle pro- und anti-apoptotische Proteine identifiziert, welche auch in ITP eine Rolle spielen könnten. Auch ist es nicht bekannt, was Apoptose in den Thrombozyten der ITP auslöst.

In Mäusen wurde gezeigt, dass TNF-α apoptotische Vorgänge wie auch eine Thromobzytopenie induziert. Diese Beobachtung deutet darauf hin, dass Zytokine einen Einfluss haben könnten auf das Einsetzten der Apoptose. Die Ziele meiner Dissertation waren (I) zu untersuchen, ob ITP Patienten bei Diagnose erhöhte apoptotische Vorgänge in ihren Thrombozyten aufweisen, (II) zu prüfen ob Thrombozyten von ITP Patienten eine beeinträchtigte Funktion haben, (III) die apoptotische Signalkaskade in gesunden Thrombozyten zu charakterisieren, und (IV) zu analysieren ob die Plasma Zytokinlevel von ITP Patienten mit der Caspase-Aktivierung, die in ITP Thrombozyten beobachtet wurde, korrelieren.

ITP Patienten wiesen bei Diagnose einen erhöhten Anteil von Thrombozyten mit aktiver Caspase-3, -8 und -9 wie auch von Mikropartikel auf. All diese apoptotischen Zeichen waren nach IVIg Behandlung normalisiert. Ausserdem hatten Thrombozyten von ITP Patienten bei Diagnose eine erhöhte Oberflächenexpression von CD63 und CD62P, eine verringerte Thrombozyten-Aktivierbarkeit und ein reduziertes Thrombingenerationspotenzial. Wir haben herausgefunden, dass in Thrombozyten nach Beginn der Apoptose, Omi/HtrA2 von Mitochondrien ins Zytosol freigesetzt wird. Zudem haben wir bestätigt, dass Mitochondrien von Thrombozyten Cytochrom c und Smac/Diablo freisetzen. In gesunden Thrombozyten konnte die Aktivierung von Caspase-3 und -9 durch eine Inhibition von Omi/HtrA2 mittels Ucf-101, einem pharmakologischen Inhibitor, reduziert werden. Wir konnten XIAP mit Embelin inhibieren und dabei apoptotische Vorgänge in Thrombozyten induzieren. Zudem fanden wir in Thrombozyten auch FADD, eine Komponente der extrinsischen apoptotischen Signalkaskade. Ausserdem stellten wir in ITP Patienten bei Diagnose nicht nur Thromozytenapoptose und eine reduzierte Thrombozytenaktivität fest, sondern auch ein erhöhtes Plasmalevel von Zytokinen der Th1 und Th2 Familie.

Zusammenfassend sehen wir, dass akute ITP Patienten erhöhte apoptotische Vorgänge in Thrombozyten aufweisen, wie auch eine reduzierte Thrombozytenaktivierbarkeit. Diese Ereignisse wurden nach einem Anstieg der

Thrombozytenzahl, verursacht durch die IVIg Behandlung, normalisiert. Ebenfalls haben akute ITP Patienten erhöhte Plasma Zytokinlevel. Wir konnten keine Korrelationen aufzeigen zwischen den analysierten Zytokinen und der Caspase Aktivierung. Deshalb ist es unwahrscheinlich, dass die von uns untersuchten Zyokine in Thrombozyten von ITP Patienten Apoptose induzieren. Eine Inhibierung von Omi/HtrA2 durch Ucf-101 in gesunden Thrombozyten bewies die anti-apoptotische Funktion von Omi/HtrA2. Eine Inhibierung von XIAP durch Embelin deutete an, dass in Thrombozyten aktive Caspase-3 und Caspase-9 durch XIAP inhibiert wurden. Diese Resultate unterlegen, dass Thrombozyten eine funktionelle apoptotische Signalkaskade haben in Gesunden wie auch in Kranken.

Indem wir die Mechanismen, welche zum programmierten Zelltod im Thrombozyten führen analysieren, könnten wir neue Einblicke in die Pathophysiologie der ITP gewinnen und neue mögliche Therapieansätze entwickeln. Eine Ausweitung der Studie könnte eventuell dazu beitragen einen Laborparameter zu identifizieren, welcher mit dem Schweregrad der Blutungen korreliert um somit besser vorauszusagen, welche ITP Patienten ein Risiko für eine Hirnblutung, bzw. eine chronische ITP zu entwickeln aufweisen.

Summary

Primary pediatric immune thrombocytopenia is a common and benign platelet autoimmune disorder. Pediatric ITP usually occurs after viral infections and is characterized by a production of autoantibodies against platelet glycoproteins. There is evidence that these autoantibodies are involved in the enhanced clearance of platelets that is observed in ITP patients. Due to the low platelet count, a typical feature of ITP is a sudden onset of petechiae in formerly healthy children. The yearly incidence of ITP is 100 cases per 1 million people whereas half of the affected patients are children. In most cases, acute ITP resolves spontaneously or after treatment within three months. However, 0.1 – 0.2% of the patients are at high risk for fatal cerebral bleeding. In around 10% the disease lasts longer than 1 year and progresses into a chronic ITP. Treatment of ITP patients with intravenous immunoglobulins (IVIg) causes an increase in platelet count. The current working model of ITP suggests that IVIg prevents the enhanced platelet clearance by blocking the Fc receptors of macrophages. In a murine model of ITP, administration of anti-platelet antibodies developed a severe thrombocytopenia. Additionally, also apoptotic events were observed in these platelets. Both thrombocytopenia and platelet apoptotic events were ameliorated by IVIg in the murine ITP model. So far it was not known whether these apoptotic events occur also in pediatric ITP.

Apoptosis is the main mechanism that regulates cellular life span but also leads to the elimination of damaged nucleated cells. Crucial mediators of the apoptotic signaling are caspases that have a proteolytic function. Caspase-8 is involved in the extrinsic and caspase-9 in the intrinsic apoptotic pathway. Activation of caspase-8 or -9 results in active caspase-3 and -7 that are effector caspases that cleave target substrates causing cell death. Active caspase-9, -3 and -7 are inhibited by XIAP, an X-linked inhibitor of apoptosis proteins. In contrast, XIAP is inactivated by the pro-apoptotic protease Omi/HtrA2, a component of the intrinsic pathway that is released from mitochondria after mitochondrial outer membrane permeabilization. Also in anuclear platelets, apoptotic manifestations such as a release of cytochrome c, an activation of caspase-3 or an increase in platelet-derived microparticles were identified. However, the presence of XIAP and Omi/HtrA2 has not been described yet in platelets. Platelet apoptosis was shown to occur also in diseases such as Bernard-Soulier syndrome or diabetes mellitus type 2. Apoptotic events seen in these diseases are often accompanied by defects in platelet function. Until now, platelet apoptosis and platelet function have not been analyzed in a well-defined pediatric ITP cohort before and after IVIg therapy. Besides, in platelets, not all pro- and anti-apoptotic proteins were identified that might as well be involved the disease of ITP. Additionally, it is not clear what the initial

apoptotic trigger in platelets of ITP patients is. In mice, TNF-α was shown to induce apoptotic events and thrombocytopenia. This observation indicated that cytokines might be involved in the onset of apoptosis. Therefore, the aims of my PhD thesis were (I) to investigate whether ITP patients at diagnosis have increased apoptotic events in platelets, (II) to study whether platelets of ITP patients show an impaired function, (III) to characterize the apoptotic signaling in healthy platelets, and (IV) to analyze if levels of cytokines in plasma of ITP patients correlate to caspase activation seen in ITP platelets.

ITP patients at diagnosis presented increased proportions of platelets with activated caspase-3, -8 and -9 as well as elevated platelet-derived microparticles. All these apoptotic manifestations were ameliorated after an increase in platelet count by IVIg treatment. Furthermore, platelets of ITP patients at diagnosis had enhanced surface expression of CD63 and CD62P compared to healthy individuals, a decreased activability of platelets and a reduced endogenous thrombin potential. In healthy platelets, apoptotic events as activation of caspase-3 and caspase-9 were decreased by inhibiting Omi/HtrA2 with its pharmacological inhibitor Ucf-101. We demonstrated that Omi/HtrA2 is released from mitochondria into the cytosol upon induction of apoptosis. We also confirmed that platelet-mitochondria release the mitochondrial proteins cytochrome c and Smac/Diablo into the cytosol. By antagonizing XIAP with its inhibitor embelin we were able to induce apoptotic events in platelets. Additionally, we found that FADD, a component of the extrinsic apoptotic signaling pathway, is expressed in platelets. Besides, ITP patients at diagnosis presented not only increased platelet apoptosis and reduced platelet activability but also elevated plasma levels of cytokines of the Th1 and Th2 family.

To conclude, acute ITP patients had increased platelet apoptotic events and reduced platelet activability that were ameliorated after an increase in platelet count caused by IVIg treatment. Furthermore, ITP patients presented elevated plasma cytokine levels. We could not show a correlation of analyzed cytokines to caspase activation thus it is unlikely that these cytokines function as an extrinsic apoptotic trigger in platelets of ITP. In healthy platelets, inhibition of Omi/HtrA2 by Ucf-101 proved the pro-apoptotic function of Omi/HtrA2 and inhibition of XIAP by embelin demonstrated that active caspase-3 and caspase-9 were inhibited. Concluding, these results highlight that platelets have a functional intrinsic apoptotic signaling pathway in health as well as in disease.

By analyzing the programmed cell death in platelets, especially its onset, we might gain new insights into the pathophysiology of ITP and reveal possible targets for additional therapeutic approaches. By including more subjects in our study, we may be able to find a laboratory parameter such as thrombin generation that correlates to the bleeding signs or the patient's history and thereby better predict which ITP patients are potentially prone for cerebral bleeding or chronic ITP; this is our future clinical research intention.

CHAPTER 1

General introduction

Platelet apoptosis in health and disease

Platelet physiology

Platelets are important players in hemostasis and blood coagulation; additionally they are involved in inflammation, thrombosis and wound healing. Hemostasis is the process that terminates bleeding, primarily mediated by the formation of blood clots characterized as blood coagulation [1]. Thrombosis is defined by a newly generated blood clot in an injured blood vessel and not only occurs in injured platelets but also in physiologically altered arteries during diseases such as heart attacks. Platelets are produced by megakaryocytes in the bone marrow [2]. Megakaryocytes arise from CD34 expressing pluripotent bone marrow stem cells [3]. Chemokines and cytokines contribute essentially for their differentiation into megakaryocytes: the granulocyte-macrophage colony-stimulating factor GM-CSF as well as interleukin IL-3 are important for the formation of megakaryocyte colonies while IL-6 is a late factor in megakaryocytes differentiation [4]. During maturation, megakaryocytes enlarge to a diameter of 100 μm and become polyploid as they lose their capacity to divide but retain their ability to replicate DNA [5]. During this process, the c-Mpl ligand thrombopoetin (TPO) stimulates megakaryoctes to increase in cell-size and ploidy. Platelets are generated by cytoplasmic extensions of megakaryocytes. These so called proplatelets contain granules and organelles and are released into the bloodstream [2]. During proplatelet formation, changes in the organization of the cytoskeleton occur caused by activation of microtubules, actin depolymerization and phosphorylation of myosin [6]. One megakaryocyte generates around 2000-3000 platelets. Following platelet release, the remaining megakaryocytes, consisting of some residual cytoplasm surrounding the nucleus, undergo apoptotic cell death [2]. A healthy platelet production results in around 1 $x10^{11}$ platelets per day and a normal platelet count varies around 150-400 $x10^{9}$/L. Megakarocytes regulate the number of circulating platelets via the hormone TPO that binds to the TPO receptor c-Mpl [7]. Platelets are discoid and have a diameter of 2-3 μm. They possess mRNA from megakaryocytes and they are capable of protein synthesis.

Platelets contribute to hemostasis by their adhesive functions and by activating coagulation mechanisms (Figure 1). Hemostasis can be stuctured into primary and

secondary hemostasis. Primary hemostasis includes the formation of a primary platelet plug at the injured vessel wall site and involves platelets, the blood vessel wall and von Willebrand Factor (vWF) [8]. The secondary hemostasis occurs simultaneously and results in the formation of a stable platelet plug by the formation of fibrin through a coagulation cascade, a process where coagulation and clotting factors play an important role as well as Ca^{2+}. A crucial step in the primary hemostasis is the activation of platelets that comprises platelet adhesion, secretion and aggregation [9] [10]. Upon vessel injury, the thrombin producing tissue factor (TF) that is present in endothelial cells, platelets and leukocytes is exposed to the bloodstream as well as collagen and vWF. The coagulation cascade is initiated by the TF. The expression of TF was shown to be upregulated in endothelial cells and monocytes by the tumor necrosis factor TNF-α or by IL-6 [11]. Platelets adhere to the subendothelial matrix via interaction of their gylcoproteins (GP) with collagen and vWF and become activated after their exposure to collagen, thrombin and other agonists. During this process collagen binds to GPVI and GPIa/IIa, vWF to GPIb and to activated GPIIb/IIIa (also known as $\alpha_{IIb}\beta_3$), and thrombin to the receptors PAR-1 and PAR-4. During platelet activation, Ca^{2+} is mobilized from the dense tubular system, an analog to the endoplasmatic reticulum in nucleated cells (Figure 2), into the cytoplasm. This mobilized Ca^{2+} activates scramblase that transports phospholipids between membrane leaflets in a bidirectional way [12]. The transmembrane protein 16F (TMEM16F) was identified as an essential component of the scramblase activity [12]. In activated platelets, TMEM16F mediates the Ca^{2+}-dependent externalization of phosphatidylserine (PS) from the inner to the outer plasma membrane leaflet [12]. This externalization of PS generates a negatively charged platelet surface that presents a suitable surface for the assembly of coagulation proteins via binding to the prothrombinase complex (Xa/Va) as well as tenase complex (IXa/VIIIa) and enhancing the activation of prothrombin [13]. Therefore, exposed PS contributes to the coagulation cascade and finally is involved in the generation of thrombin. In response to the elevated intracellular Ca^{2+} level or the thrombin signaling, platelets change their shape and form pseudopodia. Another consequence of the Ca^{2+} influx is the secretion of their granule or lysosomal contents as ADP, vWF and serotonin as well as P-selectin (CD62P) and CD63 that both become subsequently expressed at the platelet surface. Moreover, there is a conformational change of the fibrinogen receptor GPIIb/IIIa into the active form capable of binding fibrinogen. The continuous platelet adhesion to the endothelial wall leads to a constriction of the damaged vessels and to the formation of a plug. Following adhesion, platelets aggregate through the released agonists ADP and thromboxane A2 building stable fibrin clots finally resulting in blood coagulation. The tissue factor (TF) initiates a signaling cascade where coagulation factors, predominantly serine proteases, play a crucial role. The platelet surface factor Xa/Va complex cleaves prothrombin to thrombin that generates a thrombin burst while

thrombin, a serine protease, cleaves fibrinogen into fibrin [14]. Fibrinogen binds to activated GPIIb/IIIa, an important step in platelet aggregation. This activated fibrinogen receptor can be detected by PAC-1, a murine monoclonal antibody that recognizes an epitope on the GPIIb/IIIa complex of activated platelets only [15]. Soluble fragments of adhesion molecules such as the α- or dense- granule protein CD62P, the lysosomal protein CD63 or the cytosolic protein sCD40L are further released and get into circulation [16] [17]. This is an important step as sCD40L and CD62P participate in immune responses via an interaction of platelets with leukocytes or monocytes. CD40L binding to CD40 receptor is involved in the interaction of platelets with leukocytes as well as of endothelial cells with T cells [17]. CD62P on the other hand mediates adhesion of activated platelets to monocytes and neutrophils. CD63 plays an important role in complexing with integrins; a deficiency in CD63 presents as a storage pool defect as described in the Hermansky-Pudlak Syndrome [18]. Additionally, activated platelets shed plasma membrane vesicles also known as platelet-derived microparticles that have a pro-inflammatory effect and promote coagulation [19]. Strong physiological platelet agonists are collagen and thrombin. Processes occurring during platelet activation such as platelet secretion, aggregation and microparticle formation are thought to be regulated by calpains [20]. Calpain is a Ca^{2+}-dependent cysteine protease that upon platelet activation and Ca^{2+} increase cleaves cytoskeletal proteins including actin and undergoes autoproteolysis. The implication of calpains in platelet activation is considered to be initiated by integrin (GPIIb/IIIa) signaling [20].

Platelets circulate for around 10 days in the blood stream where they are cleared by the reticuloendothelial system (RES) [21] [22]. Apart from platelet clearance by the RES, platelet aging was also associated with apoptotic-like events [23] [24] [25]. Concretely, Mason et al. reported a connection between senescence of platelets and the intrinsic apoptotic signaling pathway and showed that platelet life span is determined by the balance of the anti-apoptotic factor Bcl-XL and the pro-apoptotic factor Bak [25].

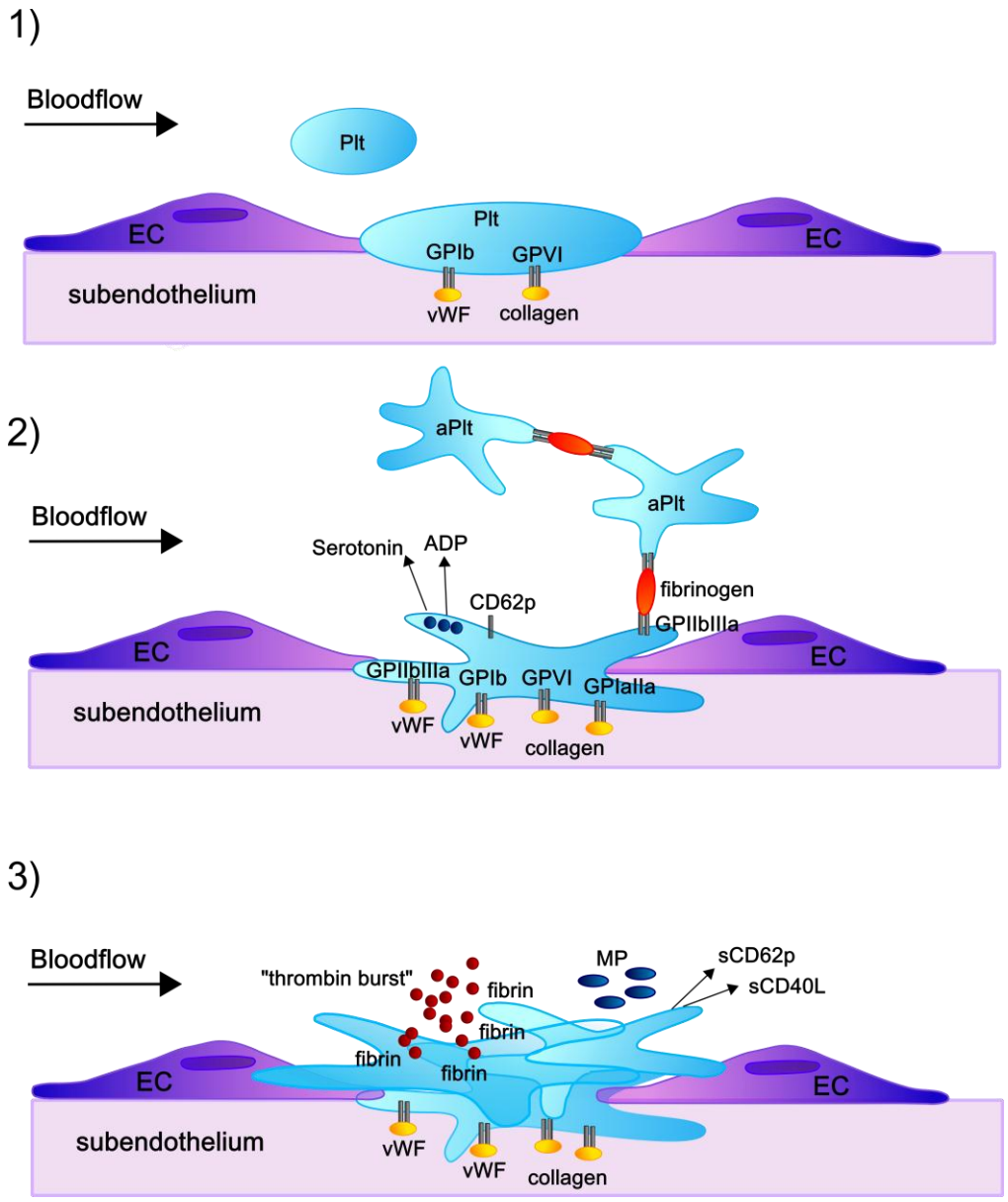

Figure 1. Platelet hemostasis

1) Adhesion of platelets (Plt) to the damaged subendothelial wall via collagen and vWF, molecules released from endothelial cells (EC), to their GP receptors. **2)** Activation of platelets (aPlt) leads to shape change, secretion of ADP, expression of proteins such as CD62P as well as to an activation of the fibrinogen receptor GPIIb/IIIa. Aggregation occurs via binding of activated GPIIb/IIIa to fibrinogen, while more activated platelets are recruited. **3)** Formation of a stable platelet aggregate and generation of a thrombin burst. Activated platelets fragment microparticles (MP) as well as soluble CD62P and sCD40L. Figure adapted from Jurk et al. [26].

The apoptotic signaling pathway of nucleated cells

Apoptosis in nucleated cells is a well-known process controlling the numbers of cells and their life-span. There are different apoptotic signaling pathways, here the extrinsic and the intrinsic pathway will be described in more detail. Cysteine-dependent ASPartyl-specific proteases, known as caspases, are the key mediators of apoptosis. They are inactive zymogens called pro-caspases that become activated upon induction of apoptosis, resulting in cleaved active fragments. The effector caspases, caspase-3 and caspase-7, act at the very end of the apoptotic signaling cascade. Caspases trigger cell destruction by cleavage of specific substrates e.g. the cytoskeletal proteins gelsolin or fodrin. The extrinsic pathway occurs via death receptors on the cell surface; for instance Fas or TNF-receptor. After activation by a ligand these death receptors recruit the cytoplasmic protein FADD leading to an activation of the initiator caspase-8 and subsequently to an activation of caspase-3 or caspase-7. The intrinsic apoptotic pathway is initiated by a depolarization of the mitochondrial membrane potential ($\Delta\psi m$) often caused by DNA damage or endoplasmatic reticulum stress. Depolarization of the mitochondria can be initiated by two different mechanisms, either by opening of the mitochondrial permeability transition pore (MPTP) in the inner mitochondrial membrane, a non-selective multiprotein pore spanning the two membrane leaflets, or directly by apoptotic proteins of the Bcl-2 family members. The formation of the MPTP occurs in response to a disruption in the Ca^{2+} homeostasis upon apoptotic stimuli and is followed by mitochondrial outer membrane permeabilization (MOMP) [27]. Bcl-2 family members such as Bad, Bid or Bax act on the outer membrane forming pores by oligomerization of Bak and Bax thus inducing MOMP [28] [29]. The extrinsic pathway is connected to the intrinsic pathway via Bid signaling: activated caspase-8 cleaves Bid and truncated Bid gets localized to the mitochondria. [30] Loss of MOMP is the onset for the release of pro-apoptotic proteins from the mitochondrial intermembrane space (IMS) as cytochrome c, Smac/Diablo or the protease Omi/HtrA2 into the cytosol. In response to a decrease in ATP levels due to cytochrome c depletion or to a free diffusion of ions between cytosol and matrix, mitochondria lose their inner mitochondrial membrane potential $\Delta\psi m$ [29] [27]. Other proteins released from IMS are the apoptosis inducing factor AIF or the endonuclease G [29]. Those proteins contribute to nuclear apoptosis by degrading and fragmenting DNA and also to chromatin condensation, a process where chromatin changes from a heterogeneous, genetically active network to an inert highly condensed form. At least two different pathways lead to nuclear apoptosis, a caspase-independent pathway including AIF or endonuclease G, and a caspase-dependent pathway via a caspase-activated DNase (CAD) [31]. Translocation of AIF from mitochondria to nuclei causes initial (peripheral) chromatin condensation and large-scale DNA fragmentation into around 50 kb fragments

whereas CAD induces advanced chromatin condensation and oligonucleosomal DNA fragmentation [31].

Following MOMP and cytochrome c release, caspase-9 is activated through the interaction of cytochrome c with Apaf-1, a process that eventually results in an activation of effector caspase-3 and -7 [32]. XIAP, an X-linked inhibitor of apoptotic proteins (IAPs), as well as the anti-apoptotic proteins of the Bcl-2 family members (Bcl-2 and Bcl-XL) are important players preventing apoptosis. Bcl-XL prevents the pro-apoptotic protein Bax from oligomerization and translocation to the mitochondria, a crucial step for MOMP. XIAP inhibits the catalytic activity of caspase-3, -7 and -9 while the pro-apoptotic proteins Omi/HtrA2 as well as Smac/Diablo are caspase activators that antagonize the inhibitory effect of XIAP [33] [34] [35]. cIAP-1 and cIAP-2 are other IAPs, binding to active caspase-3 and -7 [36]. The proteins of the IAP family are highly conserved and contain one to three baculovirus inhibitor of apoptosis protein repeats (BIR motif). These BIR motifs bind to caspases and inhibit their activity; BIR-3 was identified to specific inhibit caspase-9 while the linker region of BIR1-2 is important for caspase-3 and -7 inhibition [37]. The inhibition of caspases by IAPs is negatively regulated by the second-derived activator of caspases, Smac/Diablo, as well as the serine protease Omi/HtrA2, both proteins are released from IMS upon MOMP. Smac/Diablo competes with XIAP by binding of its BIR3 domain to the N-terminal BIR3 recognition motif of caspase-9 [38] as well as of its BIR1-2 domain to the N-terminal region of caspase-3 and -7 [39]. Omi belongs to the heat shock response serine proteases (HtrA2), is highly conserved and is sequestered in the mitochondria, protecting healthy cells from its proteolytic activity as long as mitochondria are intact. In mammalian cells, Omi/HtrA is also involved in the degradation of aberrantly folded proteins during cellular or endoplasmatic reticulum stress as well as heat shock. The BIR3 domain of Omi/HtrA2 interacts with XIAP, the same region to which also Smac/Diablo binds, and XIAP is degraded by the protease activity of Omi/HtrA2 [35]. Omi/HtrA2 was shown to induce apoptosis in human cells in a caspase-independent manner through its protease activity, and in a caspase-dependent manner via its ability to disrupt caspase-IAP interaction [40]

Apoptosis can be initiated or prevented by certain drugs, natural or artificial compounds. The anti-apoptotic effect of Bcl-XL is antagonized directly by the pharmacological inhibitor BH3 mimetic ABT-737 [41]. ABT-737 is a small-molecule inhibitor of Bcl-2, Bcl-XL and Bcl-w. ABT-737 was first analyzed in cancer cell lines and shown to improve survival and to cause regression of established tumors [41]. The inner mitochondrial matrix protein cyclophilin D (CypD) was identified as an essential regulator of the MPTP; the immunosuppressive drug cyclosporin A (CsA) interacts with CypD inhibiting formation of MPTP [28]. However, in CypD deficient mice, apoptosis was normal [42]. CypD has also been described to have a role in platelet activation and thrombosis [43]. CypD deficient

platelets present defects in PS exposure, microparticle formation and procoagulant activity upon physiological activation such as the combination of thrombin plus convulxin or thrombin plus H_2O_2 [43]. The pharmacological compound Ucf-101 inhibits the proteolytic activity of the pro-apoptotic protein Omi/HtrA2. It was demonstrated that in caspase-9 deficient mouse embryonic fibroblasts, Ucf-101 blocks the Omi/HtrA2 induced cell death [44]. Additionally, Ucf-101 was shown to have a therapeutic neuroprotective effect as in rats suffering from focal cerebral ischemia Ucf-101 reduced neurological deficits and infarct volume [45]. Embelin, an extract from the Japanese Ardisia herb, is an apoptosis activator. Embelin binds to the BIR3 domain of XIAP as does Smac/Diablo or Omi/HtrA2 thus preventing XIAP from binding to active caspase-9. In prostate cancer cells that contain high levels of XIAP, embelin selectively induced caspase-9 mediated apoptosis and inhibited cell growth [46].

Other hallmarks for apoptosis in nucleated cells include phosphatidylserine (PS) exposure that is the reorganization and loss of the asymmetry of phospholipids at the plasma membrane as well as membrane blebbing, a process that generates microparticles. Although platelets are anucleate they possess apoptotic proteins. In platelets, apoptosis was first described by Vanags in 1997 indicating the existence of an apoptotic signaling pathway [47].

Platelets have an intrinsic apoptotic signaling pathway

Since 1997 various apoptotic factors were identified in platelets on the mRNA level: caspase-1, -2, -3, -4, -6, -8, -9, p53, the death receptors DR3, DR5, DR4, TRAIL, TNF receptor p55 and RIP as well as the Bcl-2 family proteins Bcl-X, Bcl-2, Bfl-1, Bad, Bak, Bax and Mcl-1 [47] [48]. On the protein level caspase-2, -3, -7, -8 and -9 were verified in platelets [49] [50]. As activated platelets present similar events as nucleated apoptotic cells for instance PS exposure, formation of microparticles or activation of caspases, the events leading to active caspases in platelets were studied in comparison to platelet activation: The artificial agonist Ca^{2+} ionophore A23187 as well as the combination of the physiological agonists collagen and thrombin were shown to induce caspase-3 activation and PS exposure [51]; even thrombin alone triggered apoptotic events as caspase-3 and caspase-9 activation [52] [53]. However, only a high thrombin concentration (10-100 nmol/L), as eventually generated locally during coagulation, mediated platelet apoptosis [54]. In addition, thrombin was found to depolarize $\Delta\psi m$ and to generate reactive oxygen as H_2O_2, also an inducer of cytochrome c release, and lead to caspase-3 and caspase-9 activation [55]. Moreover, thrombin induced the translocation of caspase-3 and -9 from the cytosol to the actin cytoskeleton, a process that is dependent of the protein kinase C (PKC) [55]. Similar to activated platelets, platelets undergoing apoptosis shed increased microparticles, possibly an analogous event as membrane blebbing [19]. An increased production of platelet derived-microparticles was observed in response to strong activation by A23187 as well as by thrombin plus collagen [51]. It is known that in human platelets physiological agonists induce Ca^{2+} release from the dense tubular system (DTS) through the activation of PAR-1 and PAR-4 and that activation of the GPIb complex (GPIb-IX-V) results in Ca^{2+} release from the acidic stores, a Ca^{2+} pool in lysosome-related acidic organelles [56]. Lopez et al. demonstrated that DTS stress-mediated platelet apoptosis leads to activation of caspase-3, -8 and -9 [57]. In brief, Ca^{2+} mobilization is one of the first steps in platelet activation as well as in platelet apoptosis initiated by physiological agonists. Although similar events occur during platelet activation and platelet apoptosis, they are different phenomena occurring sequentially in platelet aging evidenced by platelet storage experiments [58]. It was demonstrated that PS exposure not only occurs in platelet activation but also in apoptotic platelets independently of platelet activation [59]. Two distinct pathways were shown to regulate PS exposure and therefore the pro-coagulant activity, a Ca^{2+}-dependent pathway which is caspase-independent and a caspase-dependent pathway which is Bak/Bax-dependent and is triggered for instance by ABT-737 [59].

Platelets contain mitochondria that are important for their energy metabolism and protein synthesis. An early sign of apoptosis is the collapse of $\Delta\psi m$ due to a Ca^{2+} overload in

the mitochondria via elevated intracellular Ca^{2+} levels in response to a disrupture in the Ca^{2+} homeostasis e.g. induced by agonists or drugs. Thrombin as well as the under physiological stimuli generated reactive oxygen species (ROS) H_2O_2 induce a $\Delta\psi m$ depolarization [52] [55] [60]. A total breakdown of $\Delta\psi m$ was observed upon stimulation of platelets with thrombin plus collagen [51], with the Ca^{2+} ionophores ionomycin or A23187 [61] as well as with thrombin plus convulxin, a GPVI agonist [43]. Activation of caspase-3 and PS exposure are dependent on the level of $\Delta\psi m$. The inhibitor of caspase-3, zVAD, only inhibited activation of caspase-3 while CsA inhibited both depolarization of $\Delta\psi m$ as well as PS exposure. PS exposure therefore was considered to be independent or an upstream manifestation of caspase-3 activation whereas $\Delta\psi m$ depolarization was thought to be an upstream event of caspase-9 and caspase-3 activation as well as of PS exposure [62] consistent with apoptosis in nucleated cells. PS exposure was shown to occur independently of $\Delta\psi m$ indicating that platelet apoptosis is a different mechanism than platelet stimulation [61]. To summarize, physiological as well as artificial agonists lead to $\Delta\psi m$ loss, a hallmark for the initiation of apoptosis.

Platelets contain the Bcl-2 family proteins Bcl-XL, Bax, Bak, Bad and Bid; among those Bcl-XL, Bax and Bak were studied extensively. It was shown that in resting platelets Bak was sequestered by the anti apoptotic protein Bcl-XL [63]. Stimulation of platelets with A23187 increased the expression of Bax and Bak but not of the anti-apoptotic factor Bcl-2 [64]. Thrombin was shown to induce a rapid activation of Bid and Bax followed by their translocation to the mitochondria [60]. Likewise, also the ROS H_2O_2 triggered an activation and translocation of Bid and Bax [60]. It was evidenced that platelet life span is determined predominantly by degradation of Bcl-XL during platelet aging leading to Bak triggered apoptosis followed by platelet clearance. Moreover, antagonizing the pro-survival Bcl-XL by ABT-737 resulted in thrombocytopenia, activation of caspase-3 as well as cleavage of gelsolin [25] [63]. Loss of Bak ameliorated thrombocytopenia caused by ABT-737 abolishing the effect of ABT-737 [25]. In contrast to the inhibition of an anti-apoptotic protein, activation of platelets by the collagen or thrombin mimetics CRP-XL (collagen-related peptide) or TRAP (thrombin receptor activated peptide) did not induce apoptotic signaling [63]. ABT-737-induced apoptosis promoted the generation of thrombin due to the increased PS exposure [25] [63]. Furthermore, ABT-737 interference showed defects in platelet adhesion as shedding of the platelet adhesion receptors GPIb and GPVI occurred [65]. Additionally, it was demonstrated that ABT-737 induces Bax activation and translocation [66]. In contrast to the regulation of platelet life span by Bcl-2 family proteins, the BH3-only activator proteins Bid and Bim were not required for Bax activation and mitochondrial apoptosis [66], the severe thrombocytopenia induced by loss of Bcl-XL was only dependent on Bak/Bax. In contrast, loss of either Bcl-XL, Bid or Bim did not alleviate thrombocytopenia [66]. Therefore, Bax

activation and apoptotic cell death can occur in the absence of Bid and Bim [66]. To summarize, inhibition of the anti-apoptotic protein Bcl-XL leads to platelet apoptosis observed by platelet clearance, thrombocytopenia and induction of apoptotic events.

Platelets also possess the important players of the intrinsic apoptotic pathway cytochrome c and Apaf-1 [49]. Cytochrome c and dATP-dependent caspase activation were shown to result in proteolysis of gelsolin and fodrin, proteins cleaved during apoptosis [49]. A release of cytochrome c that indicates permeabilization of the outer mitochondrial membrane was first observed in platelets aging in culture [23]. Consistent with this finding, Vogler et al. showed that apoptosis induced by ABT-737 not only mediates loss of $\Delta\psi m$ and caspases cleavage but also release of cytochrome c [63].

However, other apoptotic factors of the intrinsic pathway have not been investigated well before as Omi/HtrA2 or XIAP. It was shown that Smac/Diablo is expressed in platelets and that during storage of platelet concentrates it is released from mitochondria [67]. The interplay of the intrinsic apoptotic proteins has not been depicted precisely. Neither is it entirely elucidated whether platelets have an extrinsic signaling apoptotic pathway, as there is no evidence for the presence of death receptors or death ligands as DR3, DR5, TRAIL or TNF. Platelets express the mRNA for the death ligand TRAIL and the death receptors TNFR1, DR3, DR4, DR5 and TRADD but not for the Fas receptor nor Fas ligand [48]. All these proteins have not been detected in platelets on the protein expression level. Exclusively the decoy receptor DcR2 was found to be slightly expressed in platelets, while its expression increased during platelet storage [67]. Besides, it was reported that the death ligand TNF induced apoptotic events as thrombocytopenia, an increased production of microparticles and an activation of caspase-3, -8 and -9 [68]. By flow cytometry, the TNFR1 receptor was confirmed to be involved in the TNF signaling suggesting that death receptors and ligands have a role in platelet apoptosis [68]. However, those results where only seen in a Ca^{2+}-free, thus a non-physiological, condition.

To conclude, it is assumed that platelets have an intrinsic apoptotic signaling pathway that is initiated by a disruption in the Ca^{2+} homeostasis or the activation and translocation of pro-apoptotic proteins as Bax or by Bid signaling. Apoptosis is also triggered by physiological agonists such as thrombin or collagen leading to MOMP that causes a release of mitochondrial proteins as cytochrome c, Omi/HtrA2 and Smac/Diablo, a $\Delta\psi m$ depolarization and finally to an activation of caspase-3/7 (Figure 2). Platelet apoptosis is further induced by artificial agonists, in a Ca^{2+}-dependent manner by A23187 or Ca^{2+}-independently by ABT-737, both ways lead to $\Delta\psi m$ collapse and the release of mitochondrial proteins (Figure 3). In short, once platelets have lost their inner $\Delta\psi m$, they are determined for destruction by apoptosis. In nucleated cells, apoptosis can be diminished by the Omi/HtrA2 inhibitor Ucf-

101 and initiated by the XIAP inhibitor embelin. It remains to be elucidated whether Ucf-101 and embelin also impair platelet apoptosis (Figure 3).

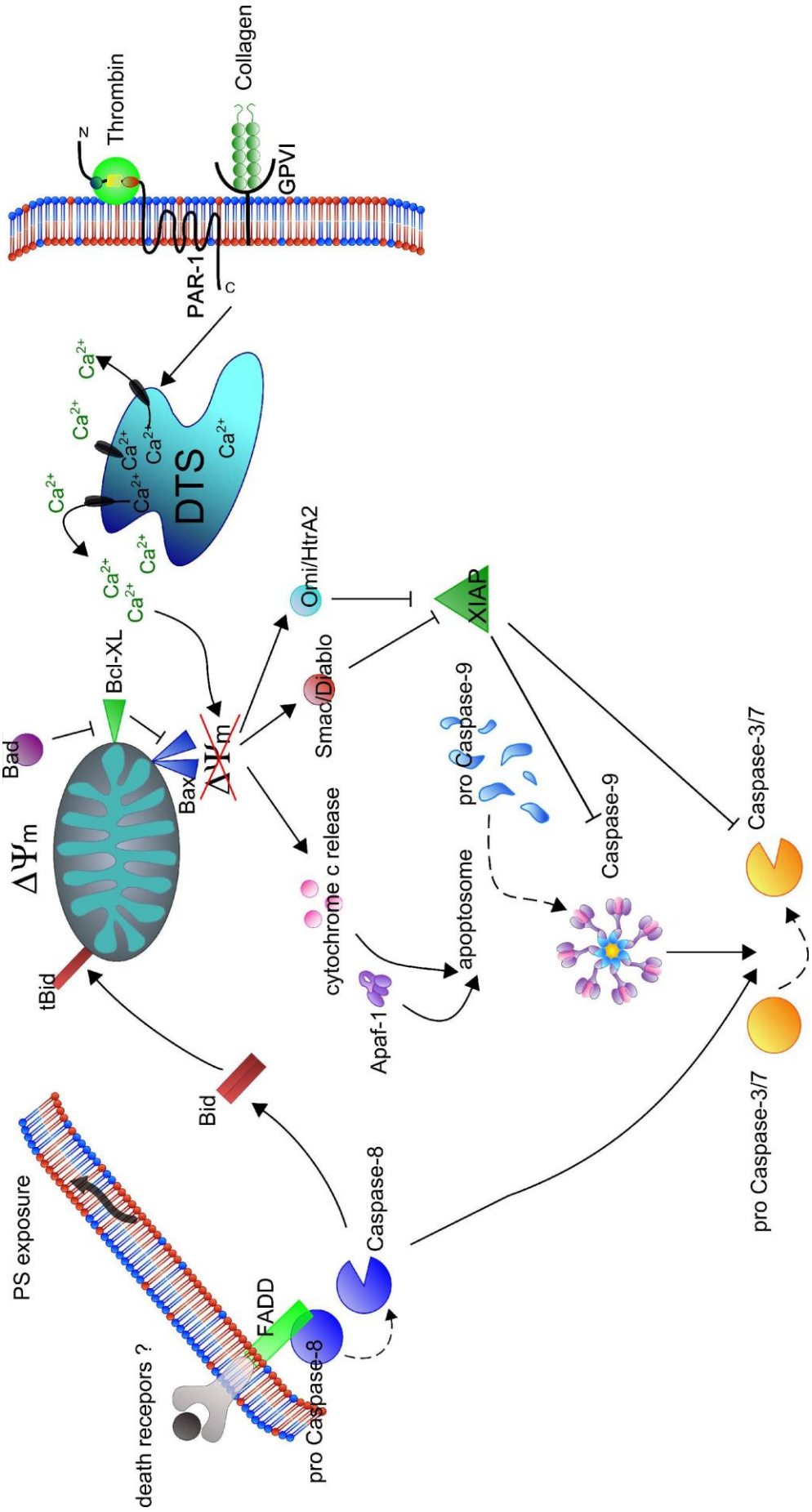
Thrombin
Collagen
GPVI
PAR-1
N
C
DTS
Ca2+
Bcl-XL
Bad
Bax
ΔΨm
tBid
Omi/HtrA2
Smac/Diablo
XIAP
cytochrome c release
pro Caspase-9
apoptosome
Caspase-9
Apaf-1
Caspase-3/7
pro Caspase-3/7
Bid
Caspase-8
PS exposure
FADD
pro Caspase-8
death recepors ?

Figure 2. Model for apoptosis in platelets. Once platelets are determined for apoptosis, apoptotic stimuli or a Ca^{2+}overload lead to a depolarization of the $\Delta\psi m$ and Bax forms pores into the outer mitochondrial membrane releasing cytochrome c, Omi/HtrA2 and Smac/Diablo from the IMS. The apoptosome assembling from released cytochrome c, dATP and Apaf-1 activates caspase-9 leading to an activation of caspase-3 or -7. The X-linked inhibitor of apoptosis (XIAP) inhibits active caspase-3, -7 and -9 and itself is inhibited by Omi/HtrA2 and Smac/Diablo. The protein Fas-associated-death-domain (FADD) is necessary for Caspase-8 activation. However, death receptors that initiate this extrinsic pathway were not discovered yet in platelets. Caspase-8 is not exclusively involved in the extrinsic pathway as it can link the extrinsic to the intrinsic pathway via truncated Bid. Physiological agonists as thrombin or collagen lead to a release of Ca^{2+} from the dense tubular system (DTS), depolarizing the $\Delta\psi m$ and releasing cytochrome c, Omi/HtrA2 and Smac/Diablo. In this step collagen (GPVI) and thrombin (PAR-1 or PAR-4) receptors are involved. PS exposure which is the translocation from PS from the inner to the outer plasma membrane occurs shortly after platelet activation as well as in platelet apoptosis upstream of caspase activation.

In addition, apoptotic events in platelets can be induced by drugs or mechanical forces. Local anesthetics as dibucaine and tetracaine promote an increase of cytosolic and mitochondrial Ca^{2+} levels inducing PS exposure, $\Delta\psi m$ dissipation, cytochrome c release, and finally an activation of caspase-9 and -3 [69]. Here, PS exposure was associated with mitochondrial apoptotic events independently of Ca^{2+} as experiments were performed in the absence of extracellular Ca^{2+} [69]. As a control for these experiments, stimulation of platelets with A23187 were also performed in the absence of Ca^{2+} that did not induce PS exposure [69]. Calmodulin (CaM) is a Ca^{2+}-sensing protein, responsible for the changes in cytoplasmic Ca^{2+} concentration, that interacts with the platelet membrane GPIb-IX-V complex and that is also considered to be involved in the regulation of apoptosis [70]. Agonists of CaM as tamoxifen or trifluoperazine have been shown to induce apoptosis in tumor cells and inhibit cell invasion and metastasis. Furthermore, it was demonstrated that CaM agonists increase the intracellular Ca^{2+} level, a depolarization of $\Delta\psi m$, caspase-3 activation and PS exposure in platelets but do not induce platelet activation [71] [72]. Furthermore, platelet adhesion and aggregation were impaired as CaM agonists interfere with the CaM binding to platelet receptors as GPVI or GPIb-IX-V [72]. In contrast, other drugs inhibit apoptosis as the immunosuppressive drug cyclosporine (CsA) that binds to the MPTP regulator cyclophilin D [72]. CsA ablolished loss of $\Delta\psi m$, activated caspase-3 as well as the formation of microparticles in A23187-stimulated platelets [72]. As for mechanical forces, apoptosis in platelets is also induced by pathologic high shear stress that are present for instance in stenotic vessels [62]. Pathologic high shear stress but not physiological shear stress induced loss of $\Delta\psi m$, caspase-3 activation, PS exposure, microparticle formation in addition to platelet activation [62].

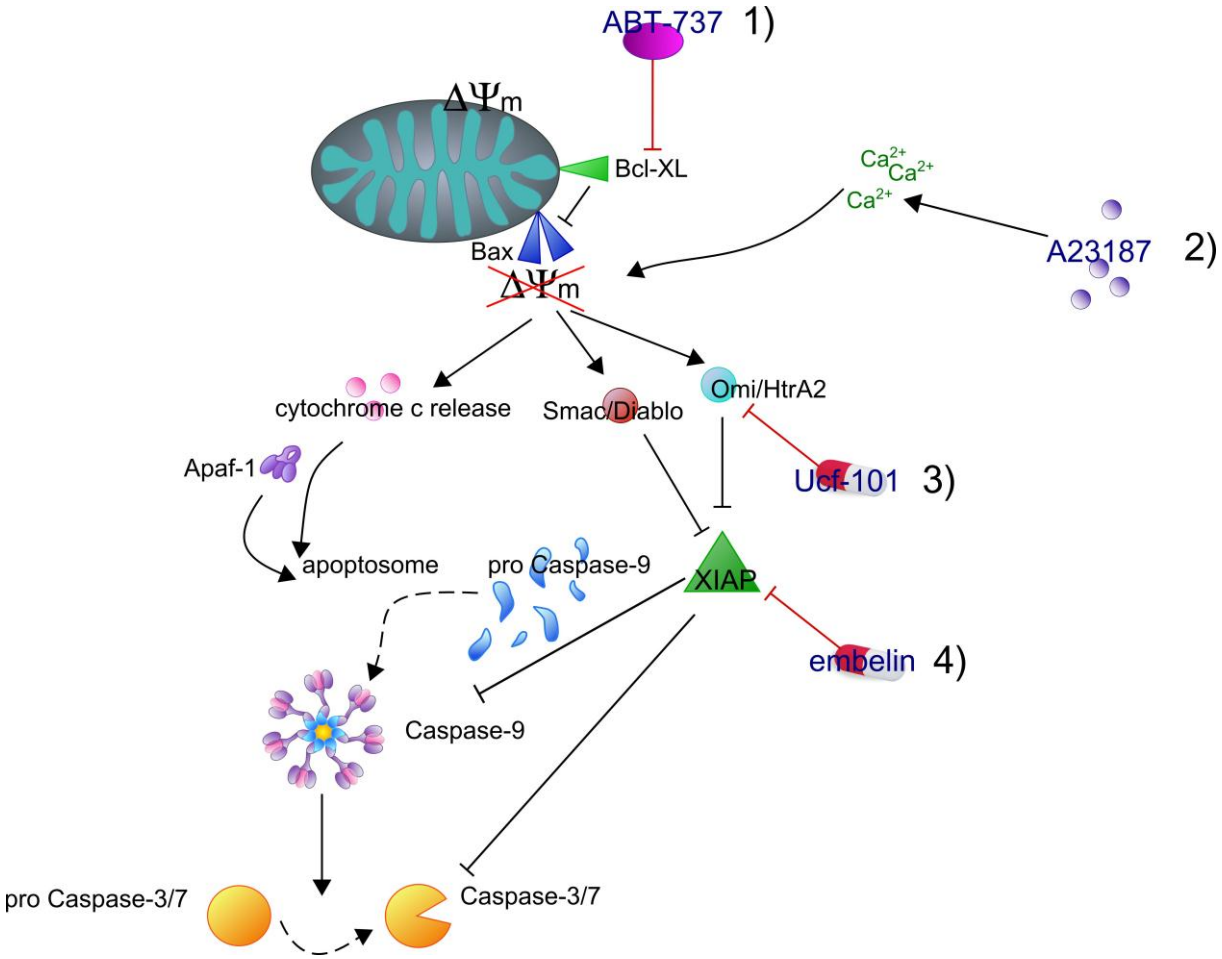

Figure 3. Model for targets of artificial agonists in the intrinsic apoptotic signaling pathway. 1) ABT-737 inhibits the anti-apoptotic protein Bcl-XL inducing depolarization of $\Delta\psi m$ releasing cytochrome c that forms the apoptosome together with Apaf-1 and activates caspase-9. Caspase-9 activates caspase-3 or -7. **2)** The Ca^{2+} ionophore A23187 forms pores in the plasmamembrane releasing Ca^{2+} that again induces loss of $\Delta\psi m$ and activates caspases. **3)** Ucf-101 inhibits Omi/HtrA2 in nucleated cells, preventing its inhibitory effect of XIAP **4)** Embelin inhibits XIAP in nucleated cells, preventing XIAP from inhibiting active caspase-3, -7 and -9.

Apoptotic events in platelet storage and senescence

It is well known that upon storage platelets lose their discoid morphology and have decreased aggregation and changes in the cell surface proteins, the so called platelet storage lesions. Loss of the intact platelet function however is a critical event in the transfusion medicine where patients suffering from a low platelet count such as leukemic patients are treated with a platelet concentrate transfusion. So far the maximum storage time of platelet concentrates has been defined to 5-7 days.

Apoptotic-like events had been associated with platelet aging; however, observations are not entirely consistent, apparently depending on the storage condition. Platelets stored in plasma-deprived medium for 18-24 hr presented apoptotic events. Specifically an increase in PS exposure, Bax and Bak expression as well as diminished $\Delta\psi m$ and a release of cytochrome c were reported [23]. Importantly, no cleaved fragments of caspase-3 could be detected suggesting that platelet death occurs in a caspase-independent way [23]. Platelets underwent a morphological condensation and monocyte-derived macrophages are supposed to be responsible for the uptake of platelets via class A scavenger receptors [23]. In contrast, a recent study of human PRP stored ex-vivo up to 5 days demonstrated a gradual increase in activated caspase-3 during platelet aging [66]. Furthermore apoptotic cell death was observed with a decline of Bcl-XL. Also expression of Bid, Bim, Bax and Bak decreased probably as no new proteins are synthesized; however, the decrease of Bak occurred later at day 5. Consistent with the finding that platelet life span is determined by the balance of the anti-apoptotic factor Bcl-XL and the pro-apoptotic factor Bak [25], Kodama et al. concluded that apoptosis is regulated by a fine balance between anti- and pro-apoptotic multidomain Bcl-2 family proteins and that spontaneous apoptosis in stored platelets occurs in a Bax/Bak-dependent way [66]. Platelet life span has also been associated with anti-platelet antibodies that promoted or inhibited activation of caspases and production of microparticles thus prolonging or shortening platelet life span [73].

An in vivo dog model with senescent circulating platelets due to the suppressed thrombopoiesis, showed evidence of a decreased $\Delta\psi m$ and increased PS exposure [24]. Platelets with shortened survival as well as senescent platelets in vivo presented increased PS exposure that was considered to be the responsible signal for platelet clearance [61]. In vivo senescent platelets further presented loss of $\Delta\psi m$ but no increased CD62P [61]. Platelets stored in buffer in vitro maintained the procoagulant surface for at least 4 hr suggesting that PS exposure may be responsible for thrombosis [74]. However, PS-exposing platelets were not cleared immediately from the circulation [75] [61] but increasing amounts of PS exposure due to stimulation with A23187 had shortened survival when injected into animals [61]. PS exposure can occur independently of $\Delta\psi m$ loss; interestingly, a

subpopulation of platelets showed loss of $\Delta\psi$m but no PS-exposure indicating that platelet aging is a different mechanism than platelet stimulation [61].

Apoptotic events were analyzed also in human platelet concentrates (PCs), stored under standard blood bank conditions. 7 days after storage of human PCs, platelets had increased PS exposure as well as CD62P [67]. The active fragment of caspase-3 was present but $\Delta\psi$m was still intact [67]. Others reported that platelet activation as CD62P occurred gradually during storage of PCs and that a decreased $\Delta\psi$m was observed only upon prolonged storage [76]. In another study of stored PCs a slight increased activation of caspase-3 was detected besides an increased expression of Bim as well as Smac/Diablo [50]. Moreover, stored PCs showed increased expression of the decoy receptor DcR2 that is an early apoptotic event [50]. Bcl-XL remained unchanged during storage as well as the pro-apoptotic proteins Bax and Bid while Bim partly translocated from the cytosol at day 11 of storage. Smac/Diablo release occurred at day 11 but no significant release of cytochrome c was observed [50]. Microparticles were shown to accumulate during 5 days of PCs storage, an apoptosis induced process without platelet activation [77] [78]. These apoptosis-induced platelet microparticles have activated GPIIb/IIIa, increased PAC-1, CD62P and PS exposure and form microparticle aggregates [77]. Therefore it was suggested that apoptosis-induced platelet microparticles promote cell macrophage differentiation and are thought to have an effect on the immune system by interacting with monocytes. Adhesion of microparticles to monocytic cells was observed, as well as the differentiation of monocytes to resident M2 monocytes and phagocytes [77]. Phagocytes are responsible for the clearance of apoptotic platelets and apoptosis-induced microparticles [77]. In an in vivo rabbit model it was shown that platelet-derived microparticles are cleared rapidly from the circulation [79]. PCs used for transfusions need to follow exact storage conditions. Only a change of temperature, especially a cold-storage followed by rewarming induced platelet apoptosis [80]. Cold storage induced a change in GPIb distribution by clustering and an association of GPIb with 14-3-3 scaffold proteins [80]. 14-3-3 proteins bind to Bad and upon association with GPIb dissociate from Bad inducing its dephosphorylation and activation, liberating Bax from Bcl-XL [81]. This GPIb-14-3-3 mediated signaling leads to translocation of Bax, depolarization of $\Delta\psi$m, release of cytochrome c and an activation of caspase-9 [80]. Rewarming reduced a second wave of apoptosis with production of thromboxane that leads to more Bax activation and depolarization of $\Delta\psi$m [80]. New approaches to reduce the risk of an infection during platelet transfusions include a pathogen reduction technology (PRT). PRT uses riboflavin or UV-light to inactivate pathogens. However, PRT treatment, affected both platelet viability and function [82]. After 5-7 days of storage of PRT treated PCs, PS exposure was increased and the pro-apoptotic proteins Bak and Bax as well as released cytochorme c and activated of caspase-3 were present [82]. Only expression of Bcl-XL was not affected by PRT [82].

Furthermore, in an earlier study it was demonstrated that UV-light induces loss of $\Delta\psi m$ [76]. So far storage of PCs has been limited to 5 days as platelet lesions occur upon longer storage. The late event of Smac/Diablo release may be associated with the observed platelet storage lesion [50].

The relation of megakaryocytes death to the generation of platelets

During a long time it was accepted that the generation of platelets is regulated by a caspase-dependent mechanism [83] [84]. Specifically the intrinsic apoptotic pathway was considered to be involved, as activation of caspases and release of cytochrome c were observed during in vitro maturation of megakaryocytes, shortly before proplatelet formation [83]. This observation was strengthened by the fact that the caspase inhibitor zVAD or an over-expression of Bcl-2 inhibited proplatelet formation [83]. However, interestingly, proplatelet formation was not induced by the apoptotic inducer staurosporine and cytoplasmic distribution of activated caspase-3 in proplatelets differed from that in senescent platelets where it is observed as DNA fragmentation and a diffuse staining pattern [83]. An increase in proplatelet production has also been observed upon induction of the extrinsic apoptotic pathway by Fas ligation [84]. In contrast to De Botton et al., Clarke et al. did not find an implication of the intrinsic pathway in proplatelet formation as neither $\Delta\psi m$ depolarization nor cytochrome c release occurred in megakaryocytes extensions and as these events only were observed in senescent platelets [84]. Furthermore, in recent in vivo studies of mice deficient for either Bak/Bax, Bcl-XL or caspase-9 as well as in vitro studies of megakaryocytes it has been demonstrated, that apoptosis is not required for platelet production, or even counterproductive [85] [86]. Activation of caspases by deleting Bcl-XL in megakaryocytes resulted in megakaryocyte apoptosis and a failure of platelet shedding [85]. In contrast, platelet production was undisturbed when Bak and Bax were deleted in megakaryocytes indicating that for platelet production a Bak/Bax-mediated apoptosis is not required [85]. It was shown that the caspase-inhibitor Q-VD-OPh did not impair proplatelet formation in contrast to zVAD, indicating that high doses of zVAD might trigger caspase-independent toxicity in megakaryocytes [85]. Besides, data showing that platelets are regulated by apoptosis was based on cell culture as well as on over-expression experiments only, but any over-expressed protein may have an impact on cellular processes and in vitro culture studies may cause errors. To conclude, Joseffson et al. evidenced that caspase activation has a negative impact on proplatelet formation and that Bak/Bax-deficient mice have an increased platelet number and life span [85]. Consistent with these findings, experiments with caspase-9-deficient mice demonstrated that caspase-9 is not essential for

the generation of megakaryocytes and platelets nor for their hemostatic functions [86]. Megakaryocytes of caspsase-9-deficient mice remained the ability to form proplatelets upon 4 days in culture assuming that caspase-9 is only required for the promotion of apoptotic death in megakaryocytes and platelets [86]. To conclude, megakaryocytes must restrain the intrinsic apoptotic signaling pathway to successively form proplatelets and shed platelets [85], while activation of the intrinsic apoptotic pathway is only mandatory for the death of megakaryocytes and platelets [85] [86]. In favor of the initial assumption of proplatelet formation is that late stage megakaryocytes produce platelets suggesting that platelet formation occurs concurrently with megakaryocytes death.

Platelet activation and apoptosis in human pathophysiology

Various diseases have been associated with platelet dysfunction, activation or apoptosis. Scott syndrome is a rare inherited bleeding disorder presenting defective platelet procoagulancy. Scott patients have a normal platelet count and structure, but their platelets fail to promote PS exposure as well as fibrin formation upon platelet activation causing bleeding events. Scott syndrome patients have deficient scramblase activity [87]; TMEM16F contributes to the scramblase complex and is essential for the Ca^{2+}-dependent PS exposure [12]. A mutation at a splice-acceptor site of the gene encoding the protein TMEM16F has been found to be responsible for its premature termination. In contrast to Scott syndrome, Bernard-Soulier syndrome (BSS) presents increased PS exposure of non activated platelets. BSS is an inherited platelet function disorder resulting in macro thrombocytopenia and bleeding. Patients with BSS present abnormality in the GPIb-IX-V receptor and a decreased interaction of both vWF and thrombin to GPIb. The low thrombin concentrations cause impaired adhesion to vessel wall and aggregation, thus bleeding. BSS was also associated with increased CD62P and apoptotic events as microparticle formation and dissipation of $\Delta\psi m$ [88]. Furthermore, abnormal platelets were discovered in patients with diabetes mellitus type 2. Those patients presented more thrombotic events probably due to hypercoagulation [89]. Diabetic rats showed a 30x increase in thrombin activity and an increased proportion of platelets with activated caspase-3, -6, -8 and -9 as well as microparticles [89]. These observed elevated microparticles are supposed to contribute to coagulation. Another disease where apoptotic events and platelet activation play a role is chronic uremia, a disease that is accompanied by kidney failure. Uremic platelets have a procoagulant activity shown by an increased thrombin generation caused by increased PS exposure [90]. Uremic platelets also presented apoptotic events as increased activated caspase-3 [90]. Furthermore, the severe form of malaria also known as cerebral malaria due to the breakdown of blood brain barrier

presented thrombocytopenia due to a reduced platelet life span. In a mouse model, cerebral malaria was associated with increased platelet-derived microparticles as well as increased proportion of platelets with activated caspases (-1, -3, -6, -8, -9) [91]. Another well-known platelet disease is immune thrombocytopenia (ITP) usually occurring upon viral infections. ITP results in a low platelet count probably due to autoantibodies generated by viruses. Infection with helicobacter pylori has been shown to be associated with ITP. The thrombocytopenia generated during H. pylori infection is due to a CD62P-dependent platelet aggregation; PS expression as well as increased microparticles were observed [92]. In a murine model it was shown that ITP is associated with apoptotic events, specifically PS-exposure, $\Delta\psi m$ loss and activation of caspase-3 [93].

Immune thrombocytopenia

Immune thrombocytopenia (ITP) is a platelet autoimmune disorder leading to a reduced circulating platelet count. At diagnosis, most ITP cases have a platelet count below 100 $x10^9$/L. ITP patients often have a preceding viral infection of approximately two weeks. The incidence of ITP is around 100 cases in 1 million people per year, half of them are children with a peak incidence of 5 years. ITP patients have mucocutaneous bleeding characterized by bruises, petechiae or purpura. Complications of ITP are chronic ITP if the disease lasts over 1 year in around 10% of the patients and cerebral hemorrhage in around 0.1 – 0.2%. ITP patients are diagnosed by a high amount of newly produced circulating platelets referred to as reticulated platelets containing a high mRNA content. [94] [95]

It is supposed that autoantibodies against platelet glycoproteins produced by viruses are responsible for the enhanced clearance of platelets in ITP. These autoantibodies of the IgG class, in particular with specificity to GPIIb/IIIa and GPIb/IX, lead to the enhanced platelet clearance (Figure 4) [95]. Subsequently, the antibody-coated platelets bind to macrophages or dendritic cells through Fc receptors and are then internalized and degraded in the spleen and liver. These antigen presenting cells additionally generate epitopes from platelet glycoproteins and express the novel peptides on their cell surface along with costimulatory help and cytokines that facilitate the proliferation of T cell clones. T cells on the other hand stimulate B cells whose immunoglobulin receptors proliferate and synthesize anti–glycoproteins too. Unfortunately, it is very difficult to diagnose ITP patients by measuring platelet autoantibodies. Cytokines were also considered to be involved in the history of ITP patients, while they were mainly measured in chronic ITP. It was shown that chronic ITP patients have increased plasma levels of the Th1 cytokines IFN-γ [96] [97], TNF-α and IL-2 [96]. Th1 cytokines are involved in inflammatory reactions and in the activation of

macrophages. Furthermore, increased levels of the Th17 cytokine IL-17 as well as IL-6 and IL-23 that are involved in the activation of Th17 cells were observed [97]. Th17 cells have been associated with inflammatory diseases and especially with autoimmune diseases. Acute ITP patients presented increased levels of GM-CSF [98], that is important in the generation of megakaryocytes.

ITP patients often recover spontaneously; however, due to the risk of fatal intracranial hemorrhage, in Switzerland most pediatric ITP patients are treated with intravenous immunoglobulins (IVIg), in other countries patients are more treated with corticosteroids or anti D-antibodies [94]. IVIg is an antibody preparation from blood donors that blocks the Fc receptors of macrophages so that platelets are not degraded anymore; anti-D antibodies also prevent platelet clearance by blocking the reticuloendothelial system (RES) and corticosteroids as oral prednisone suppress the action of the immune system. In the majority of ITP patients, platelet count increases 24 - 48 hr after administration of IVIg. ITP patients not responding to the above mentioned therapies, mainly chronic patients, are treated with splenectomy removing the primary site of platelet clearance. A further therapy is the administration of anti-CD20 antibodies as Rituximab that interfere with platelet autoantibody production eliminating the B cells by lysis. A new treatment for chronic ITP includes thrombopoietin (TPO) mimetics as Eltrompopag that increase the production of platelets by enhancing thrombopoiesis [99] [100]. Despite the low platelet count, circulating thrombopoietin (TPO) levels are mostly normal in ITP patients. It was reported that impaired platelet production is involved in some ITP cases as platelet autoantibodies may also inhibit megakaryopoiesis [101]. As TPO not only is involved in the regulation of the circulating platelets but also stimulates the differentiation of megakaryocytes, TPO mimetics leading to an increase in TPO levels may counteract this deficit of impaired megakayorcytosis. In a murine model of ITP it was shown that when anti-GPIIb antibodies are injected into mice it leads to thrombocytopenia which is ameliorated by IVIg [93] [102]. Further it was shown that murine ITP is associated with apoptotic like events as caspase-3 activation and PS exposure which are ameliorated by IVIg [93]. Administration of anti-GPIIb antibodies further induced a depolarization of the mitochondrial membrane potential ($\Delta\psi m$); however, this early sign of apoptosis was not ameliorated by IVIg suggesting that IVIg only targets downstream apoptotic effects [93].

As anti-platelet antibodies lead to thrombocytopenia and apoptotic events in mice, we wanted to investigate whether platelet apoptosis is involved in the enhanced platelet clearance that occurs in childhood ITP. The aims of this thesis were to investigate whether markers for apoptosis as well as for activation are altered in platelets from acute pediatric ITP patients. Especially we examined if an increase in platelet count in response to IVIg is associated with a decrease of markers for platelet apoptosis and activation. Besides, we

aimed to find out whether an extrinsic signal is responsible for the induction of apoptosis in acute ITP, by analyzing plasma levels of 42 different cytokines in ITP patients before and after IVIg treatment. We also studied the platelet function in ITP patients in regard to thrombin generation. To refer our findings to the autoimmune disease of ITP patients and not to the thrombocytopenia itself, we included patients with a chemotherapy-related thrombocytopenia. Furthermore, we aimed to understand the potential apoptotic signaling pathway(s) in healthy platelets. Therefore we identified apoptotic factors in platelets and inhibited or induced apoptosis in platelets by specific pharmacological compounds.

In this study, we show that ITP patients at diagnosis have increased platelet apoptosis, increased platelet activation and a reduced endogenous thrombin potential. All these manifestations were ameliorated by IVIg treatment. As well, ITP patients at diagnosis had elevated levels of cytokines of the Th1 family or Th2 family involved in B cell differentiation. We further demonstrate that platelets have a complete intrinsic apoptotic signaling pathway including the pro-apoptotic protein Omi/HtrA2 and its target inhibitor XIAP.

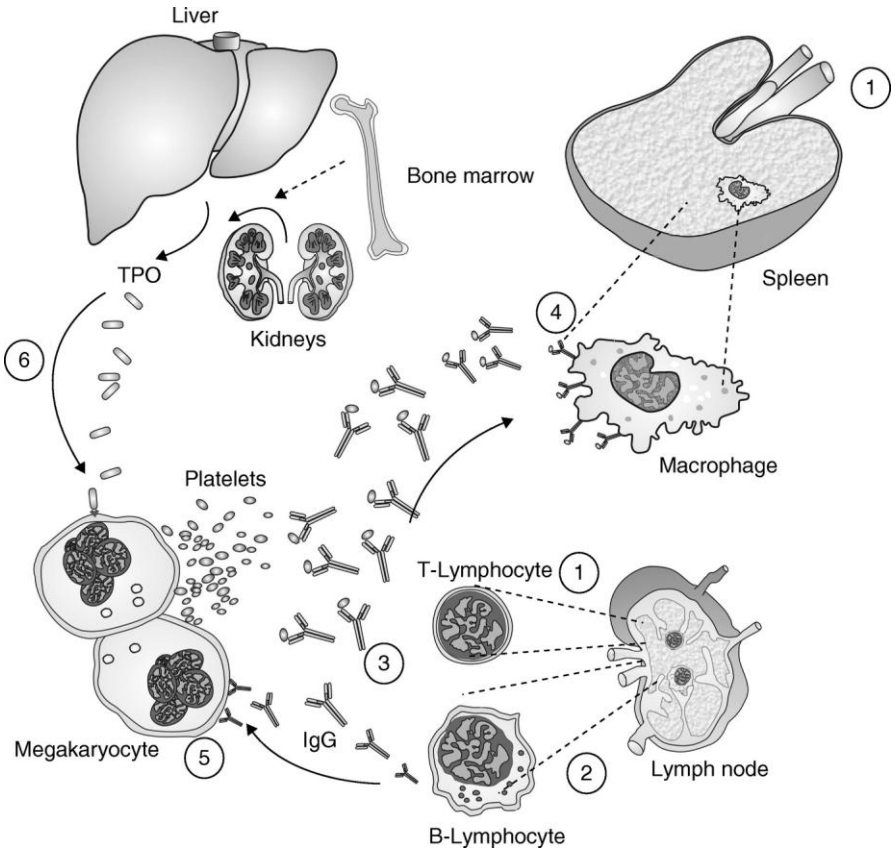

Figure 4. ITP pathophysiology and targets of therapies [103]

1) T cells lose tolerance to platelet glycoproteins, presenting platelet antigens to B cells. **2)** Stimulated B cells then produce platelet antibodies, anti-CD20 antibodies as Rituximab treatment intererferes with this step. **3)** Platelet autoantibodies bind to platelets through their fab fragment. **4)** Macrophages recognize the platelet bound autoantibodies degrading them in the spleen; this primary target for platelet clearance is removed by splenectomy. **5)** Platelet autoantibodies may also lead to a destruction of megakarycytes. **6)** TPO is constantly secreted by the liver, as well as by the kidneys and bone marrow. TPO levels vary in ITP patients; they can be similar to controls but also lower. TPO mimetics increase the platelet production.

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Outline of the thesis

In order to better understand the apoptotic signaling in platelets, we analyzed platelet apoptosis in disease and health and characterized the intrinsic apoptotic signaling pathway in human platelets. We investigated not only platelet apoptosis but also the platelet function in patients with pediatric immune thrombocytopenia (ITP), before and after treatment with intravenous immunoglobulins (IVIg).

In **Chapter two** we describe apoptotic events in children with acute ITP before and after treatment with IVIg. This study demonstrates that ITP patients at diagnosis have increased proportion of platelets with activated caspase-3, -8 and -9 as well as enhanced formation of microparticles. These apoptotic events were normalized after treatment with IVIg.

To assess if ITP patients have an impaired platelet function, we analyzed bleeding signs, platelet activability and the generation of thrombin before and after treatment of ITP patients. In **Chapter three** we present that ITP patients at diagnosis have elevated CD62P and CD63 expression, as well as a reduced thrombin potential. Further we show that IVIg reduces thrombin activation and increases the endogenous thrombin potential.

To determine further proteins of the apoptotic signaling pathway in healthy platelets, we first identified and then specifically inhibited components of the intrinsic apoptotic pathway by pharmacological compounds. In **Chapter four** we describe that platelets contain the pro-apoptotic protein Omi/HtrA2 and its target anti-apoptotic protein XIAP. Ucf-101, a specific inhibitor of the protease Omi/HtrA2 diminishes apoptotic events, while embelin, a XIAP inhibitor, induces apoptosis.

To investigate whether cytokines are involved in the apoptotic signaling of ITP as an extrinsic trigger, we describe in **Chapter five** 42 cytokines in ITP patients' plasma before and after treatment. ITP patients at diagnosis present increased plasma levels of cytokines such as TNF-α, IFN-γ, IL-2, IL-6, IL-10, IL-13 and GM-SCF.

Chapter six provides a general discussion of the work described in this thesis.

CHAPTER 2

Research Paper

Platelet apoptosis in pediatric immune thrombocytopenia is ameliorated by intravenous immunoglobulin

Jeannine Winkler*[1,2], Sabine Kroiss*[1], Margaret L. Rand[3], Imane Azzouzi[1,2], K.W. Annie Bang[4], Oliver Speer*[1,2],Markus Schmugge*[1]

*first (JW, SK) and last (OS, MS) authors contributed equally to the work

[1]*Division of Hematology and Children's Research Center, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland*

[2]*Zurich Center for Integrated Human Physiology, University of Zurich, Switzerland, University of Zurich, Switzerland*

[3]*Division of Hematology / Oncology, The Hospital for Sick Children, 555 University Ave, Toronto, ON M5G 1X8, Canada*

[4]*Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, ON M5G 1X5, Canada*

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Correspondence: Dr. Markus Schmugge, Division of Hematology, University Children's Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland

E.Mail: markus.schmugge@kispi.uzh.ch

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Summary

To evaluate the role of intravenous immunoglobulin (IVIg) in platelet apoptosis in pediatric immune thrombocytopenia, we investigated platelets of 20 pediatric patients with acute immune thrombocytopenia (ITP), before and after IVIg treatment. Healthy children with platelet counts in the normal range and children with thrombocytopenia due to chemotherapy were enrolled as controls. All ITP patients presented with platelet counts < 20 x 10^9/L and bleeding symptoms. Markers of apoptosis, including activated caspase-3, -8 and -9, phosphatidylserine (PS) exposure, mitochondrial inner membrane potential $\Delta\Psi m$, as well as platelet-derived microparticle formation, were analyzed by flow cytometry. After IVIg treatment, platelet counts increased to > 20 x 10^9/L in all patients. ITP patients had significantly increased proportions of platelets with activated caspase-3, -8 and -9, with PS exposure, and with decreased mitochondrial inner membrane potential, and demonstrated increased microparticle formation. Except for $\Delta\Psi m$, these markers for apoptosis were reduced by IVIg treatment. Platelets of children with thrombocytopenia after chemotherapy also demonstrated increased microparticle formation and decreased $\Delta\Psi m$, but no activation of caspases 3, 8 and 9 or PS exposure. In conclusion, in acute pediatric ITP, enhanced platelet apoptosis is seen at diagnosis that normalizes after IVIg treatment.

Keywords: childhood ITP, IVIg, platelet apoptosis, caspase-3, phosphatidylserine exposure

Introduction

Primary immune thrombocytopenia (ITP) is a common and mostly benign illness; children with ITP often have a history of a viral infection (Walker and Walker, 1984, Wright et al., 1996). In rare occasions, severe and even life-threatening hemorrhage can occur and currently, the majority of patients with acute primary ITP receive treatment with corticosteroids, anti-D antibody or intravenous immunoglobulin (IVIg) (Blanchette and Bolton-Maggs, 2008; Neunert et al., 2008). IVIg, an antibody preparation collected from a large number of blood donors, increases the platelet count in most patients with primary ITP.

Apoptosis is the main mechanism that regulates cell life span and the elimination of damaged or infected nucleated cells. Although platelets are anucleate, they have been observed to undergo apoptotic-like events such as collapse of the mitochondrial inner membrane potential ($\Delta\Psi m$), activation of caspases-3, -8 and -9, phosphatidylserine (PS) externalization and shedding of microparticles (Leytin et al., 2009; Leytin et al., 2008; Leytin et al., 2006a; Leytin et al., 2006b; Lopez et al., 2009; Mason et al., 2007; Rand et al., 2004; Schoenwaelder et al., 2009; Vogler et al., 2011). Recently, in a murine model of ITP, it has been shown that the profound thrombocytopenia that results from the injection of anti-glycoprotein (GP)IIb antibodies is accompanied by apoptotic-like events in the platelets; administration of IVIg was demonstrated to ameliorate the thrombocytopenia, and to inhibit late mechanisms of platelet apoptosis (Crow et al., 2003; Leytin et al., 2006b; Piguet and Vesin, 2002; Siragam et al., 2005; Song et al., 2003).

To extend these previous observations of others in an animal model of ITP to studies in ITP patients, we investigated whether enhanced apoptotic processes occur in platelets of pediatric patients with primary ITP, and whether treatment with IVIg ameliorates the enhanced apoptosis.

Materials and methods

Patients

In this prospective study, approved by the local ethics committee, 20 children (11 females and 9 males) with newly diagnosed ITP were investigated after obtaining written informed parental consent (Table 1). All patients fulfilled the criteria for primary ITP (Provan et al., 2010). The median age at diagnosis was 4.8 years (range: 2.1 – 14.4 years). The median platelet count at diagnosis was 4 x 10^9/L (range: <1 – 17 x 10^9/L).

In 13 patients, ITP was preceded by 1–3 weeks by an infectious disease: 5 children suffered from an upper respiratory tract infection; 4 had a gastrointestinal infection, including one due to *Campylobacter*; 1 child suffered from respiratory tract infection and *Norovirus* infection; 1 had a *Varicella Zoster* virus infection; and 2 patients had a febrile illness of unclear etiology. In 7 patients, there was no history of infection. The severity of bleeding symptoms at diagnosis was graded with a standardized bleeding score according to Buchanan and Adix (Buchanan and Adix, 2002) and Bolton-Maggs and Moon (Bolton-Maggs and Moon, 1997) with minor changes.

All patients were treated with an IVIg dose of 0.8 g/kg. Platelet counts were re-evaluated 12 to 24 hours after IVIg therapy. If they remained below 20 x 10^9/L, patients received an additional dose of IVIg (0.8 g/kg). Platelet counts were then re-evaluated again 12 to 24 hours later. If they remained below 20 x 10^9/L, another dose of IVIg was given. Maximally, three IVIg doses were administered.

Nineteen healthy control children, 10 females and 9 males, median age of 6.9 years (range: 0.8 – 19 years), with no history of autoimmune disease, coagulopathy, transfusion of blood products, or of ongoing medication, were recruited from the outpatient clinic after obtaining written informed parental consent. To control effects due to thrombocytopenia itself, in addition, 10 children, 4 females and 6 males, median age 10 years (range: 3.7 – 15.4 years), with chemotherapy-related thrombocytopenia (cTP), were recruited after obtaining written informed parental consent. Their median platelet count was 14 x 10^9/L (range: 3 – 50 x 10^9/L).

Table 1

Clinical and laboratory characteristics of children with primary ITP: bleeding scores and platelet count results before and after IVIg treatment are presented for each individual patient

Patient #	Age (years)	Sex	Bleeding score	Platelet count at diagnosis ($x10^9$/L)	IVIg dose (0.8 g/kg)	Platelet count after last IVIg dose ($x10^9$/L)
1	3.5	F	2.5	9	1	46
2	2.4	F	2.5	<1	3	29
3	14.2	F	3	<1	2	52
4	12.0	M	3	7	1	41
5	2.4	F	3	7	1	40
6	6.6	M	3	<1	3	42
7	4.8	F	3	7	1	59
8	4.8	M	2.5	3	2	57
9	14.4	F	2	4	1	59
10	13.9	M	2	<1	1	98
11	10.1	F	1	17	1	39
12	4.0	M	3	10	1	39
13	6.5	F	2.5	14	1	54
14	2.7	F	3	<1	2	20
15	7.8	F	2	4	1	123
16	2.5	F	2.5	2	2	45
17	2.5	M	2.5	6	1	24
18	2.8	M	2.5	8	1	24
19	5.8	M	2.5	1	1	47
20	2.1	M	2.5	<1	1	31
median	4.8	-	2.5	4	1	43.5

Materials

For flow cytometric analyses, PE-conjugated monoclonal antibodies (MAbs) to CD41a (GPIIb, clone HIP8), CD42a (GPIX, clone BEB1) and CD42b (GPIb, clone HIP1) were used, as well as APC-labeled annexin A5 (that binds to cell-surface exposed phosphatidylserine). Labelled MAbs, annexin A5, and thiazole orange were purchased from Becton Dickinson (Rotkreuz, Switzerland); fluorochrome inhibitors of caspases (FLICA) FAM-DEVD-FMK specific to active caspase-3, FAM-LETD-FMK specific to active caspase-8 and FAM-LEHD-FMK specific to active caspase-9 were from Millipore (Zug, Switzerland); the voltage-dependent, membrane intercalating dye tetramethylrhodamine-ethyl-ester (TMRE) used to analyze $\Delta\Psi m$ was purchased from Invitrogen (Basel, Switzerland). The 0.9 μm polystyrene latex marker beads used to discriminate platelets from platelet-derived microparticles were purchased from BioCytex (Marseille, France).

Blood sampling and routine testing

Venous blood samples for flow cytometric analyses were taken from ITP patients at the time of diagnosis prior to treatment, and 12 to 24 hours after the last treatment with IVIg (prior to discharge from hospital). Blood samples were taken from healthy controls when inserting a venous line and from children with cTP prior to transfusion of a platelet concentrate. Samples were collected into citrate anticoagulant (final concentration, 10.5 mM). The total volumes of the blood samples taken did not exceed 1% of the total blood volume of the patients or the controls. Blood indices including platelet counts were measured using an automated blood counter, Sysmex XE-2100 (Sysmex Digitana, Horgen, Switzerland).

Flow cytometry

Flow cytometric analyses were done using platelet-rich plasma (PRP) obtained from citrated whole blood by centrifugation at 140 g at 22 °C for 10 min. To analyze markers of platelet apoptosis, PRP was diluted 10-fold with isotonic Hepes-buffered saline with Ca^{2+} (HBS-Ca^{2+}; 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM GPRP, 2 mM Hepes, pH 7.4), containing the PE-labeled MAb to CD42b, annexin A5-APC and either FAM-DEVD-FMK, FAM-LETD-FMK, or FAM-LEHD-FMK, for analysis of active caspase-3, -8 or -9, respectively. For analysis of $\Delta\Psi m$, PRP was diluted 10-fold with HBS-Ca^{2+} containing PerCP-labeled MAb to CD42a and 100 nM TMRE. After 1 hour incubation at 22°C, samples were diluted further 10-fold with HBS-Ca^{2+} and acquired. To quantify the fraction of reticulated platelets (RP), PRP was diluted 100-fold in PBS, pH 7.4, containing 2 mM thiazole orange and the PE-labeled MAb to CD41a, incubated for 1 hour at 22°C, and acquired. For each sample, 10'000 platelets, identified as CD42a-, CD42b or CD41a-positive events, were acquired using a

FACSCalibur flow cytometer (Becton Dickinson, Rotkreuz, Switzerland). Platelet microparticles (MPs) were distinguished from intact platelets on the basis of the profile of forward light scatter vs. fluorescence, as described (Schmugge et al., 2003). The percentage of MPs was defined as the proportion of all CD42a-positive events smaller than 0.9 μm, determined using calibrated beads. Results are expressed as the percentage of platelets binding FAM-DEVD-FMK, FAM-LETD-FMK, FAM-LEHD-FMK, annexin A5, or thiazole orange. For $\Delta\Psi m$ analysis, the geometric mean fluorescence intensity (MFI) for TMRE of CD41a positive events was quantified. Data were analyzed with FCS Express (De Novo Software, Los Angeles, CA, USA).

Western blotting

Platelet proteins were separated on 12% BisTris NuPage gels (Invitrogen, Basel, Switzerland) and transferred to nitrocellulose membranes (Bio-Rad Laboratories AG, Reinach, Switzerland). Caspase-3 was detected with a monoclonal mouse IgG anti-human caspase-3 antibody (Cell Signaling Technology, Boston, MA, USA) recognizing the proform of caspase-3 (~30 kDa), and the large (~14-21 kDa) subunit of activated caspase-3, and an anti-mouse IgG coupled to horse radish peroxidase (Jackson ImmunoResearch, Newmarket, UK). Immunoreactive bands were visualized using ECL reagents (GE Healthcare, Glattbrugg, Switzerland) and autoradiography.

Statistical analyses

Statistical analysis between the 4 groups (ITP, ITP after IVIg, healthy controls, cTP) was done using Kruskal-Wallis one-way ANOVA, followed by Wilcoxon signed-rank test between pairs of medians. Correlation was assessed by the Spearman correlation coefficient. Medians were considered statistically significantly different for $p<0.05$. Analysis was done using SPSS (SPSS, Zurich, Switzerland).

Results

At diagnosis, 17 children had platelet counts below 10 x 10^9/L, and three between 10 and 20 x 10^9/L. All patients presented with bleeding symptoms, seven patients having a bleeding score of 3, twelve of 2 or 2.5 and one of 1 (Table 1).

After IVIg therapy (0.8 g/kg), platelet counts in 14 children increased to ≥20 x 10^9/L after one dose, in 4 patients after a second, and in 2 patients only after a third dose of IVIg. After the last IVIg treatment, all patients showed more than a two-fold increase in their platelet count (Table 1). Bleeding symptoms resolved in all patients; new bleeding symptoms did not occur once IVIg was given.

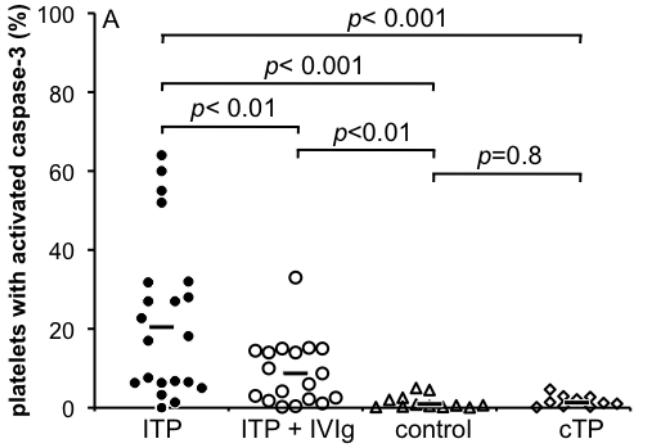
In a murine ITP model, platelet apoptosis was shown to be enhanced; caspase-3 was activated, surface PS exposure was increased and the mitochondrial inner membrane potential ($\Delta\Psi m$) was depolarized (Leytin et al., 2006b). To determine whether these observations could be extended to patients with ITP, we investigated apoptosis markers such as caspase-3, -8 and -9, as well as PS exposure, platelet-derived microparticles (MPs) and $\Delta\Psi m$ in platelets by flow cytometry. At diagnosis, a significantly higher proportion of platelets from the ITP patients contained activated caspase-3 (median 20.4%; range 1.4–64; n = 20) compared with controls (0.95%, range 0 – 5.9; n = 18) as well as with chemotherapy-related thrombocytopenic patients (cTP; 1.3%, range 0.1 – 4.6; n = 10; Figure 1A). Although in ITP the median proportion of platelets with increased caspase-3 activation was higher, not all ITP patients had platelets with activated caspase-3 even when their platelet counts were greatly decreased (Figure 1A). After IVIg therapy, the median proportion of platelets with activated caspase-3 in the patients decreased significantly to 8.7% (range 0.2 – 33; n = 19) (Figure 1A). Western blot analysis confirmed the presence of activated caspase-3 in platelets from patients before and after IVIg therapy. Activated caspase-3 was detected by Western blot only, when the proportion of platelets with activated caspase-3 as measured by flow cytometry was markedly elevated (Figure 1D). Both caspase-8 and -9 were also activated in a significantly higher proportion of ITP platelets (median 16.7 %; range 1.0 – 42.7; and 13.1%; range 1.5 – 59.6 respectively; n = 12) at diagnosis compared with healthy children (0.7%; range 0.04 – 2.3 and 0.4; range 0.03 – 2.16; n = 11) and cTP patients (1.8%; range 0.9 – 3.8; and 1.8%; range 0.6 – 2.9; n = 11; Figure 1B & C). As with caspase-3, proportions of platelets with activated caspase-8 and -9 also decreased significantly after IVIg therapy to a median of 2.5% (range 0.1 – 10.0; n = 8) and 7.1% (range 0.7 – 15; n = 8), respectively.

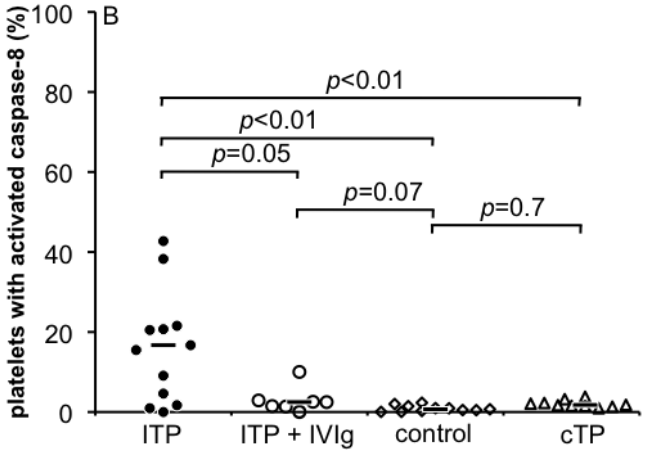
The proportion of platelets with surface-exposed PS at ITP diagnosis (median 15.3%; range 0.9 – 38.5; n = 20), was significantly higher than in controls (1.5%; range 0.6 – 5.5; n = 19) and in children with cTP (1.7%; range 0.6 – 4.9; n = 10). After IVIg therapy, the

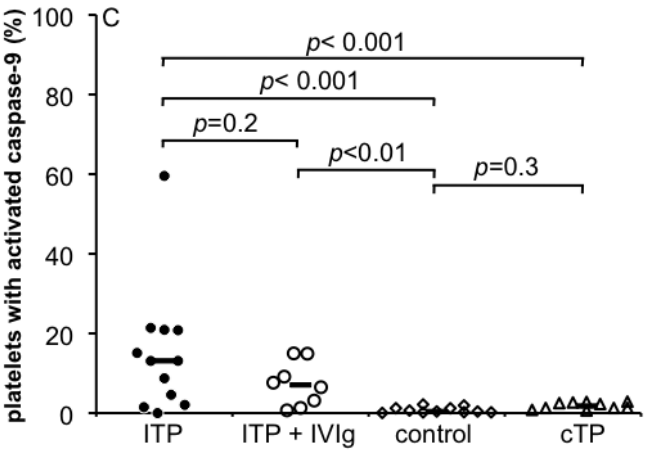
proportion of platelets with surface-exposed PS decreased non-significantly to a median of 7.2% (range 1.3 – 20.3; n = 19; Figure 2A.)

In a group of children with ITP (n = 12) platelets double stained for both surface exposed PS and activated caspase-3 were studied simultaneously (see material and methods). Only a median of 35% (range 3 – 95) of platelets with increased PS exposure also had activated caspase-3, and a median of 3.2% (range 0.5 – 46) of platelets had activated caspase-3 without PS exposure. Furthermore the proportion of platelets showing PS exposure only (median 9.4%; range 0.5 – 28), as well as the proportion of platelets showing activated caspase-3 only (median 3.2%; range 0.5 – 46), were both elevated at diagnosis and decreased after IVIg (3.8%; range 1.4 – 14.8; and 0.85%; range 0 - 36.8, respectively). These findings indicate that surface PS exposure is not necessarily associated with activated caspase-3 in platelets from ITP patients.

Additionally, the mitochondrial inner membrane potential ($\Delta\Psi m$) of platelets as analyzed by TMRE fluorescence tended to be lower at diagnosis of ITP compared to healthy controls. $\Delta\Psi m$ did not recover after IVIg therapy. Although not significantly different, $\Delta\Psi m$ in platelets from healthy children was higher compared to all other patient groups (Figure 2B).


A
platelets with activated caspase-3 (%)
100
80
60
40
20
0
p< 0.001
p< 0.001
p< 0.01
p<0.01
p=0.8
ITP
ITP + IVIg
control
cTP


B
platelets with activated caspase-8 (%)
100
80
60
40
20
0
p<0.01
p<0.01
p=0.05
p=0.07
p=0.7
ITP
ITP + IVIg
control
cTP


C
platelets with activated caspase-9 (%)
100
80
60
40
20
0
p< 0.001
p< 0.001
p=0.2
p<0.01
p=0.3
ITP
ITP + IVIg
control
cTP

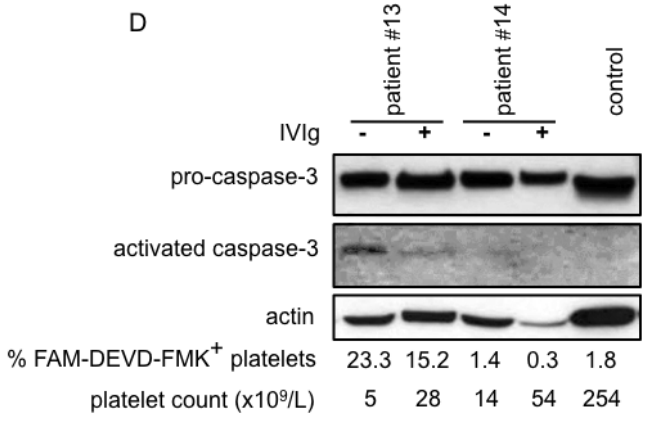

D
patient #13
patient #14
control
IVIg
-
+
-
+
pro-caspase-3
activated caspase-3
actin
% FAM-DEVD-FMK+ platelets
23.3
15.2
1.4
0.3
1.8
platelet count (x10⁹/L)
5
28
14
54
254

Figure 1. Platelet caspase activation is increased in children with primary ITP and decreased after IVIg therapy. (A-C) Percentages of platelets with activated caspase-3, as determined by FAM-DEVD-FMK fluorescence **(A)**, with activated caspase-8 as determined by FAM- LETD -FMK fluorescence **(B)** and percentages of platelets with activated caspase-9, as determined by FAM- LEHD -FMK fluorescence **(C)**. The proportion of platelets with activated caspases for each child is shown and the median for each group is indicated by a horizontal line. **(D)** Western blotting of pro-caspase and activated caspase-3 levels from individual patients (patient 13 and 14) and control. Below the blots, flow cytometry results of platelets with activated caspase-3 (% FAM-DEVD-FMK$^+$ platelets), and the respective platelet counts are indicated as well. An overall comparison indicated significant differences between groups (caspase-3 $p < 0.001$; caspase-8 $p < 0.01$; caspase-9 $p < 0.001$).

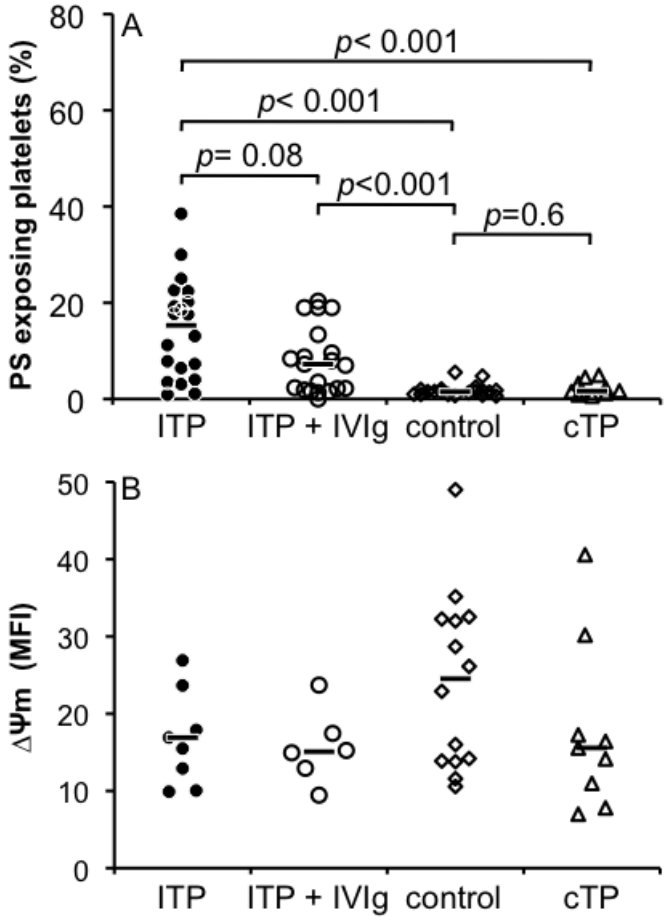


Figure 2. Loss of both platelet membrane phospholipid asymmetry (PS exposure) and mitochondrial inner membrane potential in ITP is not significantly ameliorated after IVIg. Percentage of platelets exposing PS, as determined by annexin A5 binding **(A)**. Mean fluorescence intensity (MFI) of TMRE determining the mitochondrial inner membrane potential ($\Delta\Psi m$) in platelets **(B)**. Analyzed parameters for each child are shown and the median for each group is indicated by a horizontal line. An overall comparison indicated significant differences between groups for PS exposure ($p < 0.05$) but not for $\Delta\Psi m$ ($p = 0.4$).

Finally, at diagnosis, platelet-derived MPs were significantly elevated compared with controls (median 9.0%; range 3.3 – 23.1; n = 20 vs. 5.6%; range 0.8 – 13.2; n = 19), and decreased to normal values after IVIg treatment (5.5%; range 1.5 – 13; n = 18). Interestingly, platelet-derived MPs in cTP patients were as high as in ITP at diagnosis (Figure 3).

In keeping with previous observations (Kokawa et al., 1991; Saxon et al., 1998; Strauss et al., 2010; Thomas-Kaskel et al., 2007), the median proportion of young, reticulated platelets as well as median platelet size was significantly elevated (median 14.9%; range 2.4 – 27.8; and median 32.8 FSC; range 13 – 109; n = 20) in our ITP study cohort, compared to that in healthy children (1.1%; range 0.6 – 3.0; and 19.0 FSC; range 13.6 – 24.1; n = 19) as well as cTP (1.8%; range 1.1 – 4.2; and 21.4 FSC; range 14.0 – 33.1; n = 8). There was no correlation between reticulated platelets and caspase-3 activity (r = 0.21, p = 0.4).

After IVIg therapy, the proportion of reticulated platelets as well as platelet volume in the patients decreased significantly to 2.9% (range 0.7 – 16; n = 18) and to 25.9 FSC (range 12.0 – 42.2; n=18), respectively (Figure 4). However, the absolute amount of reticulated platelets in ITP (median 0.6 $x10^9$/L; range 0.002 – 3.6; n = 19) doubled after IVIg (1.2 $x10^9$/L; range 0.3 – 9.8, n = 18), recovering towards control levels (2.1 $x10^9$/L; range 0.8 – 6.7; n = 15). The observed increase in the absolute number of reticulated platelets suggests that IVIg therapy might also enhance thrombopoiesis, in keeping with recent observations by others (Barsam et al., 2011).

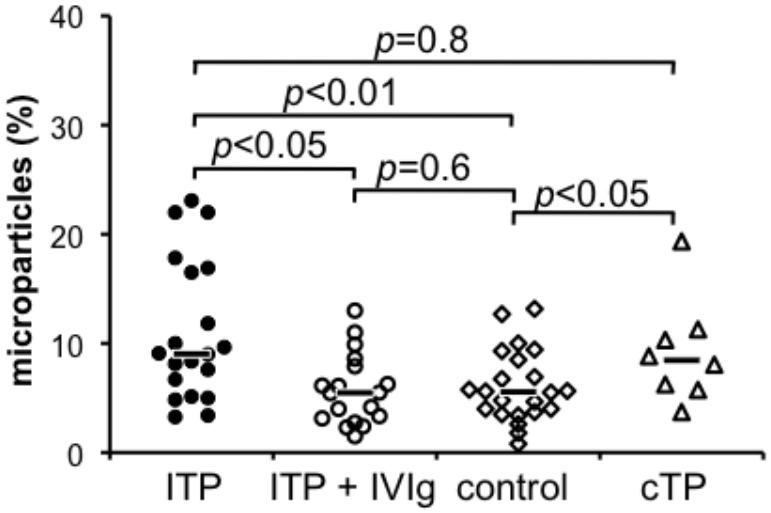


Figure 3. The proportion of platelet-derived microparticles is increased in ITP and normalizes after IVIg therapy. Percentage of platelet-derived microparticles were defined as the proportion of CD42a-positive events <0.9 μm. The proportion of microparticles for each child is shown and the median for each group is indicated by a horizontal line. An overall comparison indicated significant differences between groups ($p < 0.01$).

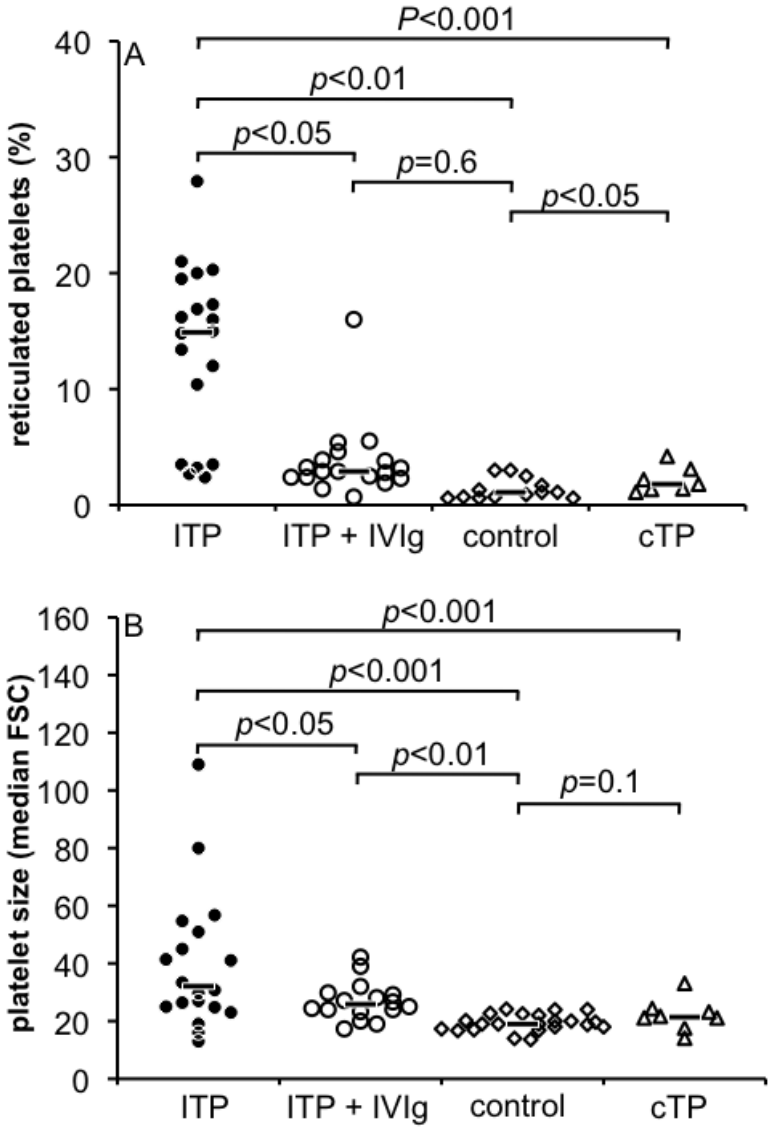


Figure 4. Reticulated platelet count and platelet size in childen with primary ITP pre- and post-IVIg therapy. Percentages of reticulated platelets, as determined by thiazole orange staining **(A)**. Platelet size, as determined by forward scatter **(B)**. The proportion of reticulated platelets and the platelet size for each child are shown and the median for each group is indicated by a horizontal line. An overall comparison indicated significant differences between groups (reticulated platelets $p < 0.001$; platelet volume $p < 0.001$).

Discussion

In this study, we investigated apoptotic markers in a clinically well-defined group of children with very low platelet counts, increased proportions of reticulated platelets and increased platelet size that presented with bleeding symptoms including petechiae, large hematomas, mucosal bleeding and epistaxis - all typical signs of primary ITP. Further, we compared these results in ITP to those from healthy children and children with cTP.

In platelets from primary ITP patients we demonstrated the presence of elevated apoptosis markers – activated caspase-3, caspase-9, and caspase-8; PS exposure; and microparticle formation; as well as decreased $\Delta\Psi m$. After IVIg therapy, the clinical symptoms of ITP subsided with the increase in platelet counts, whereas the proportions of platelets with activated caspase-3, -8 and -9 as well as the propotions of platelet-derived microparticles, decreased. PS exposure decreased, but the decrease was not statistically significantly. In contrast, $\Delta\Psi m$ did not recover after IVIg therapy. Children with cTP also demonstrated increased microparticle formation and decreased $\Delta\Psi m$, but activation of caspases-3, -8 and -9 and enhanced PS exposure were not found in this group.

In nucleated cells, hallmarks of apoptosis are morphological changes such as membrane blebbing and chromatin condensation. Additional well-established signs of apoptosis are activation of caspase-3, -8 and-9, dissipation of $\Delta\Psi m$ and release of factors from the mitochondrial inner membrane space, such as cytochrome c (Galluzzi et al., 2009). In the anucleate platelets, we detected caspase-3, -8 and -9 activation, dissipation of $\Delta\Psi m$ and platelet-derived MPs originating from membrane blebbing; all of these parameters are easily measured by flow cytometry. However cytochrome c release, which can only be analyzed by Western blot after sub-cellular fractionation or by confocal microscopy, was not considered for investigation because the small volumes of pediatric blood samples, especially samples from thrombocytopenic patients, contained too few platelets for these analyses.

It should be noted that there were some patients that had low proportions of platelets with activated caspase-3 or PS exposure, or low proportions of platelet-derived microparticles before IVIg treatment, comparable with control levels, while their platelet counts were low and their proportion of reticulated platelets was increased, indicating a heterogeneity in the apoptotic phenotype in ITP. Interestingly, the patients with low proportions of platelets with activated caspase-3 were not necessarily those with low proportions of PS-exposing platelets or with low proportions of microparticles, indicating a further heterogeneity in the apoptotic phenotype.

The apoptotic events described in platelets in the murine model of ITP occurred as a result of injection of anti-GPIIb antibodies (Leytin et al., 2006b), thus, in this model, the

antibody type and titre were both well controlled. In our patients, it is likely that the anti-platelet antibodies were either anti-GPIIb, -GPIb or –GPIIb/IIIa antibodies (Aref et al., 2009), and the titres would have been different among patients. This may contribute to the heterogeneity seen in the various apoptotic markers in the platelets from our pediatric ITP patients.

The observed apoptotic events in platelets from ITP patients might contribute to the thrombocytopenia. The increased PS exposure on ITP platelets could lead to their clearance from the circulation. In addition the increased activation of caspase-3 might lead to the disintegration of platelets and microparticle formation after cleavage of caspase-3 target proteins. However, cTP patients also showed elevated microparticle formation but without increased proportions of platelets with activated caspases and PS exposure. We speculate that in cTP, elevated microparticles originate from endothelial activation, chemotherapy related toxicity or previous platelet transfusions (Cauwenberghs et al., 2006; Morel et al., 2008). Also $\Delta\Psi m$ seemed to be dissipated in ITP as well as in cTP. Previous studies have demonstrated that exogenous thrombin is also able to induce apoptotic events; however this always seem to be accompanied by a strong mitochondrial membrane depolarization (Lopez et al., 2007).

In this prospective study of children with primary ITP, we observed elevated signs of platelet apoptosis, specifically caspase-3 activation, PS exposure and microparticle formation, which were ameliorated by IVIg administration as the thrombocytopenia improved, similar to findings in a murine ITP model (Leytin et al., 2006b).

In further support of Leytin et al, also in pediatric ITP IVIg did not inhibit platelet $\Delta\Psi m$ dissipation. With these findings Leytin et al concluded that IVIg does not affect upstream breakdown of $\Delta\Psi m$ in platelets, but does inhibit downstream apoptotic events, caspase-3 activation and PS exposure (Leytin et al., 2006b). In our study additionally platelet caspase-8 and caspase-9 activation were investigated: not only activation of caspase-3, but also of caspase-8 and -9 were ameliorated by IVIg. Therefore we conclude that in platelets IVIg not only inhibits executioner caspase-3 and PS exposure, both downstream of the extrinsic death signaling and intrinsic mitochondrial disintegration pathways, but in addition the data presented in our study indicate that IVIg also inhibits caspase-8 activation, downstream of the cell-surface death receptors, and caspase-9, that is at the crossroad of extrinsic and intrinsic apoptosis signaling pathways (Hengartner, 2000).

Currently, the pathway that initiates the apoptotic events in ITP platelets and which results in its amelioration after IVIg administration is unknown; it appears to be distinct from that of platelet activation, confirming the finding of an apoptosis-dependent pathway leading to activation of caspase-3, -8 and -9 or PS exposure, independent of P-selectin exposure (Leytin et al., 2008; Schoenwaelder et al., 2009; Vogler et al., 2011). In addition, it remains

unclear if our findings are specific for acute ITP where they could result from an increased pro-platelet formation in response to the peripheral platelet destruction (De Botton et al., 2002). In contrast, in chronic ITP, resistance to platelet apoptosis has been observed with less pronounced caspase activation; this could be a consequence of the abnormal megakaryocyte development that has been described (Nugent et al., 2009; Wang et al., 2010). Further studies are necessary to investigate these mechanisms.

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Authorship and Disclosures

OS, SK, JW, MS: designed the study; JW, OS, IA: performed the experiments, JW performed the experiments of Figure 1B, 1C, 1D and 2B and parts of Figure 1A, 2A, 3 and 4; OS, JW, SK: analyzed data; SK, MS: attended and enrolled the patients and collected clinical data; KWAB, MLR: provided essential advice and expertise with platelet testing and flow cytometry; and OS, MLR, SK, MS, JW: wrote the manuscript. The authors have no conflicts of interest to declare.

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CHAPTER 3

Original Article

Improved thrombin activation of platelets and endogenous thrombin potential in pediatric patients with immune thrombocytopenia after IVIg treatment

Jeannine Winkler*[1,2], Sabine Kroiss*[1], Oliver Speer[1,2], Markus Schmugge[1]
* authors contributed equally to the work

[1]*Division of Hematology and Children's Research Center, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland*
[2]*Zurich Center for Integrated Human Physiology, University of Zurich, Switzerland, University of Zurich, Switzerland*

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Correspondence: Dr. Markus Schmugge, Division of Hematology, University Children's Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland
E.Mail: markus.schmugge@kispi.uzh.ch

Summary

To assess which ITP patients are prone for high bleeding risk and in which patients treatment is dispensable due to low bleeding risk, we investigated platelet function in acute pediatric immune thrombocytopenia (ITP) before and after intravenous immunoglobulin (IVIg) treatment. We analyzed platelets and plasma of 23 ITP patients for platelet activation, response to thrombin stimulation as well as thrombin generation. Healthy children with platelet counts in the normal range and children with thrombocytopenia due to chemotherapy were enrolled as controls. All ITP patients presented with platelet counts < 20 x 10^9/L and bleeding symptoms. Platelet surface expression of the activation markers CD62P and CD63 as well as the conformational change in the glycoprotein GPIIb/IIIa were analyzed by flow cytometry. Furthermore, the endogenous thrombin potential was measured by a thrombin generation assay. ITP patients at diagnosis presented increased proportions of platelets with CD62P and CD63 and a decreased endogenous thrombin potential. Additionally, thrombin-induced activation of platelets was reduced in acute ITP shown by decreased expression of CD62P, CD63 and activated GPIIb/IIIa. After IVIg treatment, platelet counts increased to > 20 x 10^9/L in all patients. Thrombin-induced activation as well as the low endogenous thrombin potential were ameliorated by IVIg in ITP patients. To conclude, after IVIg treatment of pediatric ITP patients we observe an improved thrombin activation of platelets and an increased endogenous thrombin potential.

Keywords: pediatric ITP, IVIg, platelet activation, endogenous thrombin generation

Introduction

Primary pediatric immune thrombocytopenia (ITP) is a common and mostly benign illness; children with ITP often have a preceding viral infection [1] [2]. ITP is a platelet autoimmune disorder leading to a reduced circulating platelet count. ITP patients mostly recover spontaneously but in rare occasions fatal intracranial hemorrhage occurs. Also around 10% of the patients develop into a chronic ITP when the disease last longer than 12 months after diagnosis. Treatment of ITP patients includes intravenous immunoglobulins (IVIg), anti D-antibodies or corticosteroids [3] [4]. IVIg, an antibody preparation collected from a large number of blood donors, increases the platelet count and reduces bleeding symptoms in most ITP patients.

It is suggested that ITP is caused by autoantibodies binding to glycoprotein (GP) complex GPIIb/IIIa or GPIb/IX [5] resulting in an enhanced platelet clearance. There is evidence that these anti-platelet antibodies can either inhibit or activate platelets and alter platelet function [6]. Platelet activation leads to a change in the platelet morphology and to the conversion of the fibrinogen receptor GPIIb/IIIa into a functional fibrinogen receptor that binds fibrinogen, a process required for normal platelet aggregation [7]. This activated and exposed GPIIb/IIIa can be detected by the monoclonal antibody PAC-1 [8]. During platelet activation, fusion of granule or lysosomal membranes occurs exposing and expressing P-selectin (CD62P) [9] as well as CD63 [10] on the platelet surface. A first step upon damage of blood vessels is the expression of the tissue factor (TF) into the bloodstream that initiates a coagulation signaling cascade. Two different pathways are involved, the extrinsic pathway including TF, factor VII and V and the intrinsic pathway that among others consists of factor VIII and factor X. The platelet surface factor Xa/Va complex cleaves prothrombin to thrombin that generates a thrombin burst while thrombin cleaves fibrinogen into fibrin [11]. This generation of thrombin can be measured by a thrombin generation assay that determines the endogenous thrombin potential (ETP). ETP determines the time that thrombin has been active in a plasma sample and also the generated amount of thrombin [12].

We wanted to investigate whether by analyses of the ETP in plasma of ITP patients we will be able to better define the bleeding risks. To assess whether ITP patients have an impaired ETP, we measured the ETP in ITP patients before and after IVIg treatment as well as in chronic ITP patients. Recently we showed that at initial presentation pediatric ITP patients present enhanced phosphatidylserine (PS) exposure as well as platelet apoptotic events e.g. activated caspases and increased formation of microparticles [13]. PS exposure is a sign of platelet apoptosis as well as activation and it is well accepted that two distinct pathways regulate procoagulant activity [14]. All these apoptotic manifestations were ameliorated by an increase in platelet count after IVIg treatment. Earlier studies

demonstrated that platelets are activated in chronic ITP shown by increased CD62P expression [15] [16].

To extend these previous observations we wanted to analyze whether platelet function such as platelet activability is impaired in acute ITP patients. To determine whether platelet activation or thrombin generation is altered in pediatric ITP and ameliorated by IVIg we investigated markers for platelet activation as well as the endogenous generation of thrombin before after IVIg treatment. To further study platelet activability of ITP platelets we analyzed apoptotic markers as activated caspase-3, -8, and -9 in platelets stimulated by thrombin or the Ca^{2+} ionophore A23187. In this study we demonstrated that ITP patients at diagnosis have increased platelet activation, reduced thrombin-induced platelet stimulation as well as a reduced ETP.

Materials and methods

Patients

In this prospective study, approved by the local ethics committee, 23 children (13 females and 10 males) with newly diagnosed ITP were investigated after obtaining written informed parental consent (Table 1). All patients fulfilled the criteria for primary ITP [17]. The median age at diagnosis was 4.2 years (range: 1.6 – 14.4 years). The median platelet count at diagnosis was 6 x 10^9/L (range: <1 – 17 x 10^9/L) and after treatment with IVIg 41 x 10^9/L (range 20 – 123 x 10^9/L). 3 patients had signs of a respiratory tract infection without fever at the time of diagnosis, one of them presented with urtikaria. Two patients had a 1-2 weeks preceding respiratory tract infection with fever and one without fever. Two patients were still under antibiotic treatment because of streptococcal angina. In 3 patients, ITP was preceded by 2–4 weeks by a viral gastroenteritis. One patient presented with diarrhea and fever two months ago and one patient had diarrhea 2 weeks ago. One patient had a febrile infection and presented vomiting and one patient had fever 5 days before diagnosis. One patient had an influenzal infection two weeks before diagnosis and one patient presented with a 4 weeks preceding varicella. In 8 patients, there was no history of infection. All patients had a very low platelet count median 6 x 10^9/L (range <1 – 17 x 10^9/L) at diagnosis. The severity of bleeding symptoms at diagnosis was graded with a standardized bleeding score according to Buchanan and Adix [18] and Bolton-Maggs and Moon [19] which was modestly adapted. Bleeding scores were 2 to 3 with most patients suffering from large hematoma and multiple petechiae. 6 patients presented with mucosal bleeding, 2 of them with severe epistaxis and hematemesis.

All acute ITP patients were treated with IVIg at a dose of 0.8 g/kg body weight and showed a rise in platelet count above 20 x 10^9/L in the next 24 to 48 hr, accompanied by declining bleeding symptoms. From these patients blood was investigated at this diagnosis and after 24-48 hr. Patients were followed during one year. After 6 months 15 patients were in remission with stable normalized platelet counts without further treatment. One patient had persistent ITP, two a relapsing ITP and 5 patients developed into a chronic ITP.

We included 13 patients who suffered from a chronic ITP (Table 2) after obtaining written informed parental consent. These chronic ITP patients had a median platelet count of 51 x 10^9/L (range <1 – 195 x 10^9/L) (Table 2). The median age was 10.4 years (range 2.6 - 16.4). Two patients were under treatment at the time of blood collection, one under rituximab and one under prednisone treatment.

22 healthy control children, 11 females and 11 males, median age of 7.6 years (range: 0.8 – 19 years), with no history of autoimmune disease, coagulopathy, transfusion of blood products, or of ongoing medication, were recruited from the outpatient clinic after

obtaining written informed parental consent. To control effects due to the thrombocytopenia itself, in addition, 13 children (7 females and 6 males) with a median age of 10.3 years (range: 3.8 – 15.4 years), 12 patients with a chemotherapy-related thrombocytopenia and one with an aplastic anemia (cTP), were recruited after obtaining written informed parental consent. Their median platelet count was 16 x 10^9/L (range: 3 – 53 x 10^9/L).

Table 1.

Clinical and laboratory characteristics of children with primary ITP: bleeding scores and platelet count results before and after IVIg treatment are presented for each individual patient. BS indicates blood sampling.

Patient #	Age (years)	Sex	Bleeding score	Platelet count at BS ($x10^9$/L)	# of IVIg doses (0.8 g/kg)	Platelet count after last IVIg dose ($x10^9$/L)
1	2.4	F	3	7	1	40
2	4.0	M	2	10	1	39
3	6.5	F	2.5	3	1	54
4	4.2	F	3	1	1	22
5	5.8	M	2.5	1	1	47
6	2.1	M	2	<1	1	31
7	1.6	F	2	2	1	38
8	2.8	M	3	14	1	28
9	2.4	F	2.5	<1	3	29
10	14.2	F	3	<1	2	52
11	6.6	M	3	<1	3	42
12	4.8	F	3	7	1	59
13	4.8	M	2.5	3	2	57
14	14.4	F	2	4	1	59
15	13.9	M	2	<1	1	98
16	2.7	F	3	<1	2	20
17	7.8	F	2	4	1	123
18	3.5	F	2	2	2	45
19	2.5	M	2.5	6	1	24
20	3.8	M	2.5	8	1	24
21	12.0	M	3	7	1	41
22	10.1	F	1	17	1	39
23	3.5	F	2.5	9	1	46
median	4.2	-	2.5	6	1	41

Table 2.

Clinical and laboratory characteristics of children with chronic ITP: Bleeding scores, platelet counts and proportion of platelets with activated caspase-3, caspase-9 and caspase-8 are presented for each individual patient. BS indicates blood sampling.

Patient	Age	Sex	Bleeding score	Platelet count at BS	Platelets with activated			Treatment at BS
					caspase-3	caspase-9	caspase-8	
#	(years)			($x10^9$/L)	(%)	(%)	(%)	
1	2.6	F	2	2	9.3	9.7	12.3	Rituximab
2	12.9	M	1	< 1	11.8	15.6	7.7	-
3	4.4	M	1	195	0.2	0.4	0.2	-
4	12.0	M	1	11	4.1	3.9	9.4	-
5	10.4	F	1	52	0.6	2.4	1.8	-
6	7.8	M	1	51	2.9	6.3	5.1	-
7	6.6	F	1	143	nd	nd	nd	Prednisone
8	7.1	F	2	3	1.0	3.7	3.0	-
9	16.4	F	2	30	nd	nd	nd	-
10	13.5	M	1	60	2.6	7.2	7.0	-
11	14.1	F	1	126	0.1	0.8	0.5	-
12	15.8	F	0	51	1.6	0.3	0.6	-
13	5.1	F	2.5	3	nd	nd	nd	-
median	10.4	-	1.0	51	2.1	3.8	4.0	-

Materials

For flow cytometry PerCP-conjugated anti CD42a (glycoprotein (GP) IX, clone BEB1), APC-conjugated anti CD62P (clone AK-4), PE-conjugated CD63 (clone H5C6), as well as FITC-conjugated PAC-1 (clone PAC-1) were used. Fluorochrome inhibitors of caspases (FLICA) FAM-DEVD-FMK specific to active caspase-3, FAM-LETD-FMK specific to active caspase-8 and FAM-LEHD-FMK specific to active caspase-9 were from Millipore (Zug, Switzerland). Labeled mAbs were purchased from Becton and Dickinson (Rotkreuz, Switzerland).

Blood sampling and routine testing

Venous blood samples for flow cytometric analyses were taken from ITP patients at the time of diagnosis prior to treatment, and 12 to 24 hours after the last treatment with IVIg (prior to discharge from hospital). Blood samples were taken from healthy controls when inserting a venous line and from children with cTP prior to transfusion of a platelet concentrate. Samples were collected into citrate anticoagulant (final concentration, 10.5 mM). Blood indices including platelet counts were measured using an automated blood counter, Sysmex XE-2100 (Sysmex Digitana, Horgen, Switzerland).

Flow cytometry

Flow cytometric analyses were done using platelet-rich plasma (PRP) obtained from citrated whole blood by centrifugation at 140 g at 22°C for 10 minutes. To analyze markers of platelet activation, PRP was diluted 10-fold with isotonic Hepes-buffered saline with Ca^{2+} (HBS-Ca^{2+}; 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM GPRP, 2 mM Hepes, pH 7.4), containing PAC1-FITC, CD42a-PerCP, CD63-PE and CD62P-APC. For analysis of active caspase-3, -8 or -9, FAM-DEVD-FMK, FAM-LETD-FMK or FAM-LEHD-FMK were used respectively in combination with CD42b-PE. Measurements were done before and after thrombin stimulation of platelets with 1 U/ml thrombin as well as by 3 μM calcium ionophore A23187 in PRP for 1 hr. For each sample, 10’000 platelets, identified as CD42a-positive events, were acquired using a FACSCalibur flow cytometer (Becton Dickinson, Rotkreuz, Switzerland). Data were analyzed with FCS Express (De Novo Software, Los Angeles, CA, USA).

Thrombin generation

Thrombin generation (ETP) was measured with Technothrombin TGA kit (Technothrombin TGA kit, Techonoclone, Vienna, Austria) on an Infinite M200 microplate fluorescence reader (Tecan, Männerdorf, Switzerland) with a specially adapted software (Technoclone, Vienna, Austria), according to the manufacturer's instructions and as

previously reported [20]. The concentration of generated thrombin was registered over time, resulting in a thrombin generation curve. From this curve total amount of thrombin generated (area under the thrombin generation curve (AUC)) was determined.

Statistical analyses

Statistical analysis between the 4 groups (ITP, ITP after IVIg, healthy controls, cTP) was done using Kruskal-Wallis one-way ANOVA, followed by Wilcoxon signed-rank test between pairs of medians. Correlation was assessed by the Spearman correlation coefficient. Medians were considered statistically significantly different for $p<0.05$. Analysis was done using SPSS (SPSS, Zurich, Switzerland).

Results

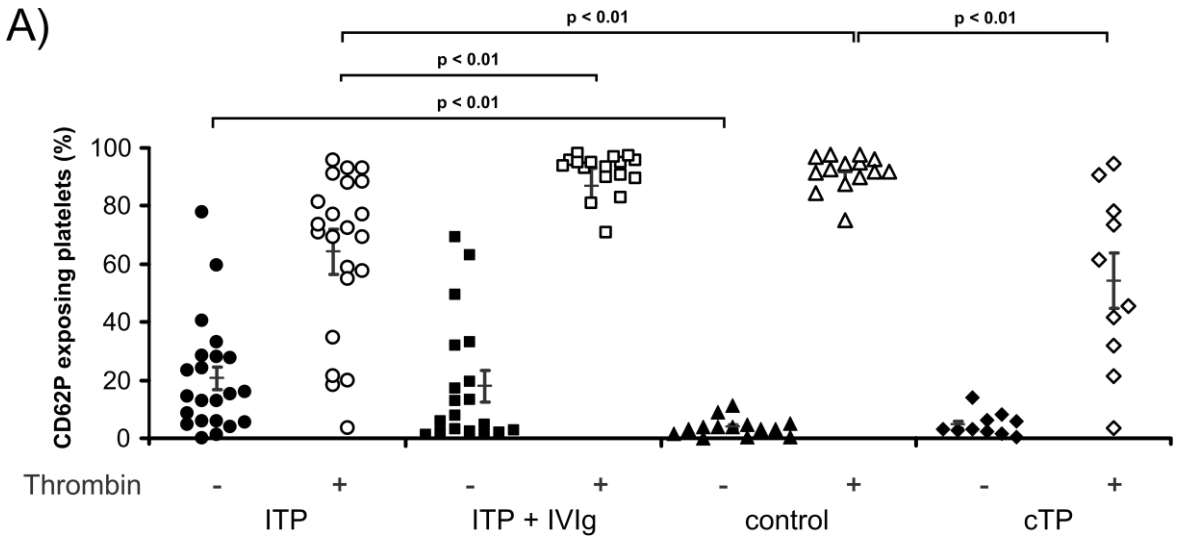
At diagnosis, 20 children with acute ITP had platelet counts below 10 x 10^9/L, and 3 between 10 and 20 x 10^9/L. All patients presented with bleeding symptoms, 8 patients having a bleeding score of 3, 7 of 2.5, 7 of 2 and 1 of 1 (Table 1). After IVIg therapy (0.8 g/kg), platelet counts in 17 children increased to ≥20 x 10^9/L after one dose, in 4 patients after a second, and in 2 patients only after a third dose of IVIg. After the last IVIg treatment, all patients showed more than a two-fold increase in their platelet count (Table 1). Bleeding symptoms resolved in all patients; new bleeding symptoms did not occur once IVIg was given. We included 13 patients with chronic ITP (Table 2) that had a platelet count of 51 x 10^9/L, 3 of them presented almost a normal platelet count of above 100 x 10^9/L One patient had no bleeding symptoms while 8 patients having a bleeding score of 1, 3 of 2 and 1 of 2.5 (Table 2).

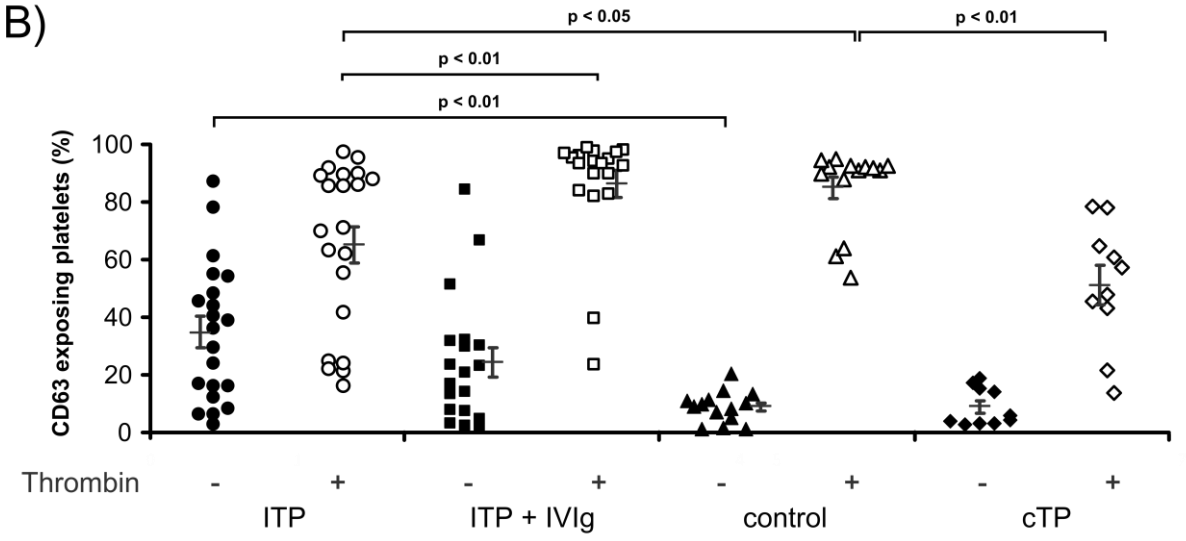
Recently we showed that at initial presentation pediatric ITP patients have enhanced platelet apoptotic events such as activation of caspases, PS exposure and formation of microparticles [13]. Those apoptotic manifestations were ameliorated after treatment with IVIg. In the present study we examined whether the same ITP patients also present impaired platelet activation or thrombin generation. To analyze whether platelet activation is impaired in pediatric ITP we investigated platelet activation markers such as P-selectin (CD62P), CD63 and PAC-1 by flow cytometry with and without stimulation by thrombin before and after treatment with IVIg. To determine the amount of generated thrombin in ITP plasma we analyzed the endogenous thrombin generation potential in acute ITP patients at diagnosis before and after treatment as well as in chronic ITP patients.

Platelets of childhood ITP patients at diagnosis presented an increased surface expression of CD62P (20.09 ± 4.3%) and CD63 (34.4 ± 5.5%) compared to cTP patients (CD62P: 4.7 ± 1.3%; CD63: 8.8 ± 2.1%) and healthy children (CD62P: 3.8 ± 0.8%; CD63: 10.6 ± 2.1%) (Figure 1A & B). Thrombin stimulation significantly enhanced the surface expression of CD62P as well as of CD63 in ITP patient's platelets (CD62P: 63.8 ± 6.2%; CD63: 64.0 ± 6.5%) as well as in cTP platelets (CD62P: 54.2 ± 9.6%; CD63: 51.2 ± 6.8%), however, values were lower compared to healthy children (CD62P: 91.6 ± 1.6%; CD63: 85.2 ± 3.8%). After IVIg treatment expression levels of CD62P as well as of CD63 were reduced in ITP patients, similar to the values of healthy children meaning normalized. The expression of CD62P in ITP patient's platelets after therapy was 18.0 ± 4.9% and increased to 86.9± 5.1% after thrombin treatment; of CD63 24.4 ± 5.1% that increased to 86.3 ± 4.6%.

Thrombin-induced PAC-1 binding, thus activation of GPIIb/IIIa, increased after stimulation of platelets with thrombin, in patients with ITP or cTP and controls. However, PAC-1 binding was reduced in ITP patients (33.5 ± 6.3%) as well as in cTP patients (16.6 ±

4.9%) compared to healthy controls (67.3 ± 6.5%) (Figure 1C). After treatment of ITP patients with IVIg, thrombin-stimulated platelets showed an increased binding of PAC-1 (53.9± 7.3%), similar to control levels. PAC-1 levels of resting platelets in ITP (2.6 ± 0.9%) and after IVIg therapy (3.5± 2.8%) were neither significantly different from control levels (1.0 ± 0.4%) nor from cTP patients (0.5 ± 0.1%).


A)
p < 0.01
p < 0.01
p < 0.01
p < 0.01
CD62P exposing platelets (%)
100
80
60
40
20
0
Thrombin
-
+
-
+
-
+
-
+
ITP
ITP + IVIg
control
cTP


B)
p < 0.05
p < 0.01
p < 0.01
p < 0.01
CD63 exposing platelets (%)
100
80
60
40
20
0
Thrombin
-
+
-
+
-
+
-
+
ITP
ITP + IVIg
control
cTP

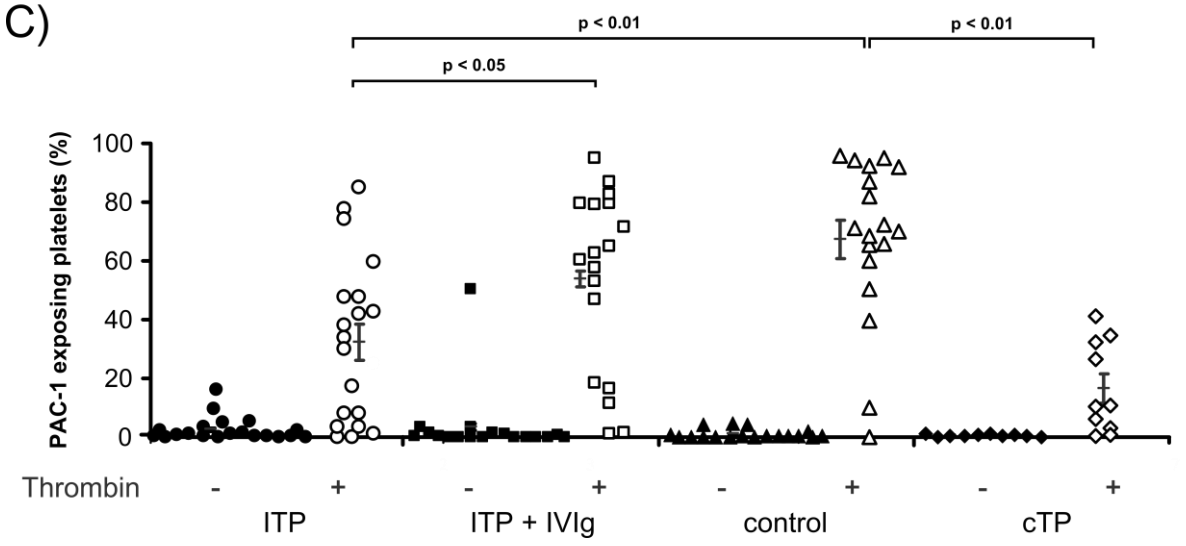

C)
p < 0.01
p < 0.01
p < 0.05
PAC-1 exposing platelets (%)
100
80
60
40
20
0
Thrombin
-
+
-
+
-
+
-
+
ITP
ITP + IVIg
control
cTP

Figure 1. ITP patients at diagnosis have impaired platelet activation. P-selectin (CD62P), CD63 and PAC-1 expression in platelets from ITP patients at diagnosis and after IVIg treatment compared to healthy children and children with cTP. Measurements were performed in the absence and presence of platelet stimulation by thrombin. Platelet activation markers were measured by flow cytometry. Patients with acute ITP present increased surface expression CD62P that is ameliorated by IVIg **(A)**. Patients with acute ITP have increased expression of the lysosomal protein CD63 that is ameliorated by IVIg **(B).** Thrombin-induced PAC-1 binding is reduced in ITP and cTP and increases after IVIg treatment **(C)**. Data are presented as mean ± SEM. Black symbols: without thrombin, white symbols: after thrombin stimulation. Circles indicate ITP patients at diagnosis, squares ITP patients after IVIg treatment, triangles healthy children and rectangle children with a chemotherapy-related thrombocytopenia. $p < 0.05$ was considered statistically significant.

Acute ITP patients showed significantly lower ETP compared to healthy children (Figure 2A). The median amount of the generated thrombin at diagnosis of ITP was 152 nM (range: 18 - 401 nM). After IVIg treatment, although platelet count did not completely normalize, ETP increased to 267 nM (range: 120 – 415 nM) comparable to values seen in healthy children (370 nM, range: 85 – 673 nM). One month after IVIg treatment, patients presented again a decreased thrombin generation as values were at 194 nM (range: 97 – 295 nM) (data not shown, n = 14). These patients also presented a low platelet count of 9 x 10^9/L (range: 3 – 274). Furthermore, 3 patients developed into a chronic ITP. There was a significant correlation observed ($p < 0.001$) between ETP and platelet count (all study subjects included: $r = 0.45$). For platelet activation markers, no correlation was seen between ETP and CD62P or CD63 expression with and without thrombin stimulation. Interestingly, cTP patients had not a reduced thrombin generation: 242 nM (range; 185 – 786 nM). We also analyzed the ETP in platelets of 13 chronic ITP patients. These patients presented a reduced platelet count of 51 x 10^9/L while 3 patients had nearly a normal platelet count between 100 and 200 x 10^9/L (Table 2). Chronic ITP patients had a significantly reduced ETP (143 nM, range: 31 – 477) compared to controls that was similar to acute ITP patients at diagnosis (Figure 2B).

To determine if chronic ITP patients also have increased proportion of platelets with activated caspases as we have detected in acute ITP patients in a recent study [13] (Chapter 2), we analyzed active caspase-3, caspase-8 and caspase-9 by flow cytometry in platelets of chronic ITP patients (Table 2). We found that chronic ITP patients present a less pronounced activation of caspase-3 at the time of blood collection (median 2.1 %; range 0.2 – 11.8, n = 10) (Table 2); compared to control (0.95 %, range 0 – 5.9; n = 18) and acute ITP (median 20.4 %; range 1.4 – 64; n = 20) as was determined before (Chapter 2). Both caspase-8 and -9 were also increased in chronic ITP patients, while capase-9 was statistically significant ($p < 0.05$) (median 4.0%; range 0.2 – 12.3, n = 10 and 3.8%; range 0.3 – 15.6, n = 10) (Table 2); compared to control (0.95%, range 0 – 5.9; n = 18) and acute ITP (median 20.4%; range 1.4 – 64; n = 20) as determined in the study of Chapter 2. Two chronic ITP patients that both had a very low platelet count presented increased activated caspases, while one patient was under treatment at the time of blood collection and the other was not (Patient Nr 1 and 2).

In Figure 1 we showed that ITP patients at diagnosis have reduced thrombin-induced platelet activation observed by a decreased expression of platelet markers. To determine whether thrombin also negatively influences activation of caspases in acute ITP, we investigated active caspase-3, -8 and -9 in thrombin-stimulated ITP platelets. Additionally, we also analyzed A23187-stimulated platelets, as the Ca^{2+} ionophore A23187 is more potent to induce apoptotic events in healthy platelets than thrombin. In Table 3 we show that caspase activation by thrombin or by A23187 in platelets of ITP patients is not impaired in most patients (apart from patient Nr 5) compared to the reduced activation of CD63, CD62P and PAC-1 by thrombin shown in Figure 1. In Table 3A & B we describe the proportion of platelets in acute ITP patients as well as in controls. In contrast to controls, ITP patients at diagnosis have already increased proportions of platelets with activated caspase-3, -8 and -9 in resting platelets. To better depict the data of Table 3A & B, we determined the fold increase in ITP and control (% thrombin-stimulated platelets divided by % resting platelets; % A23187-stimulated platelets divided by % resting platelets) of activated caspases in Table 3C & D. We have not evaluated yet the data for ITP patients after IVIg treatment. The fold increase in ITP patient's platelets after thrombin stimulation was 1.8 (range 0.4 – 3.3) for activated caspase-3 and similar to control (2.6, range 0.6 – 10.0). The fold increase in A23187-stimulated ITP patients' platelets was 2.7 (0.8 – 5.7) for activated caspase-3 and significantly lower ($p < 0.01$) than in control (32.9, range 4.2 – 60.5). The fold increase in thrombin-stimulated platelets of ITP patients was 1.6 (range 0.1 – 5.5) for activated caspase-9 and similar to control (2.4, range 0.1 – 13.7). The fold increase in A23187-stimulated ITP patients' platelets was 2.1 (0.5 – 9.2) for activated caspase-9 and significantly lower ($p < 0.01$) than in control (7.3, range 3.2 – 104.1). The fold increase in thrombin-stimulated platelets of ITP patients was 1.5 (range 0.4 – 3.8) for activated caspase-8 and similar to

control (1.5, range 0.8 – 9.2). The fold increase in A23187-stimulated platelets of ITP patients was 1.9 (0.5 – 4.9) for activated caspase-3 and significantly lower ($p < 0.01$) than in control (8.6, range 4.5 – 23.5). The acute ITP patients included in Table 3 is a subgroup of the ones included in the previous study of Chapter 2. To summarize, activation of control platelets by A23187 is much higher than activation of acute ITP patient's platelets shown by fold increases. Of these 10 patients we have complete data for activated caspase-3, -9 and -8.

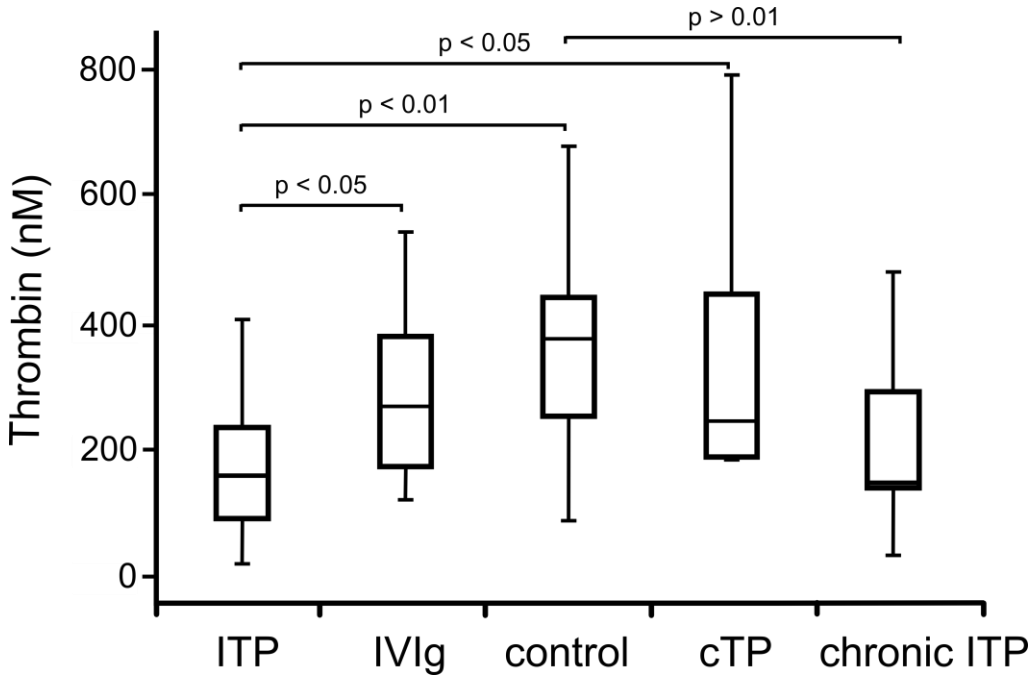


Figure 2. ITP patients have a reduced endogenous thrombin generation potential (ETP). ETP was measured in platelet poor plasma (PPP) after addition of tissue factor according materials and methods. ETP of acute ITP patients before and after diagnosis was compared to healthy children, cTP children and children with a chronic ITP. Box plots represent the generated thrombin levels (nM). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively; the horizontal bar across the box indicates the median and the ends of the vertical lines indicate the minimum and maximum data values $p < 0.01$ - 0.05. n = 11 for ITP, n = 12 for IVIg, n = 16 for control, n = 7 for cTP and n= 13 for chronic where n indicates the number of patients.

A)

ITP	Age	Sex	platelets with activated caspase-3			platelets with activated caspase-9			platelets with activated caspase-8		
Patient #	(year)		resting (%)	Thrombin (%)	A23187 (%)	resting (%)	Thrombin (%)	A23187 (%)	resting (%)	Thrombin (%)	A23187 (%)
1	2.7	F	31.8	40.0	51.7	59.6	67.8	63.8	38.3	72.0	54.8
2	8.8	F	6.5	10.1	30.8	20.8	39.6	36.4	21.6	40.7	47.8
3	3.5	F	18.2	37.2	78.2	20.9	73.4	49.6	42.7	45.7	74.3
4	2.5	M	6.3	8.3	17.7	8.7	7.6	18.2	9.1	15.4	21.8
5	2.8	M	6.8	3.0	5.7	13.4	1.1	6.8	17.0	6.1	11.2
6	7.4	M	22.7	51.7	43.4	15.1	68.7	43.7	20.5	51.9	43.6
7	5.8	M	1.4	3.7	3.9	4.6	4.5	42.5	4.6	4.2	5.5
8	2.1	M	7.6	20.7	17.6	13.1	29.0	18.3	20.7	26.7	10.5
9	1.6	F	5.1	4.7	12.8	2.2	11.8	18.4	3.5	13.1	16.9
10	2.8	M	8.8	29.0	50.8	12.5	15.5	26.9	13.8	13.7	55.7
median	2.8	-	7.2	15.4	24.2	13.3	22.2	31.7	18.8	21.1	32.7

B)

control	Age	Sex	platelets with activated caspase-3			platelets with activated caspase-9			platelets with activated caspase-8		
Patient #	(year)		resting (%)	Thrombin (%)	A23187 (%)	resting (%)	Thrombin (%)	A23187 (%)	resting (%)	Thrombin (%)	A23187 (%)
1	19.0	M	0.8	6.4	26.3	1.9	3.1	27.8	2.3	3.3	25.2
2	4.1	M	0.1	0.7	3.8	0.2	1.0	6.5	0.5	0.4	2.5
3	6.8	F	0.6	1.6	2.7	1.2	1.8	3.9	0.7	1.4	3.2
4	7.6	F	0.3	2.1	12.0	0.4	0.9	2.3	1.5	2.2	6.5
5	15.8	M	0.4	1.2	15.0	2.2	0.3	7.5	1.0	0.8	3.8
6	2.9	F	0.2	0.5	11.5	0.4	1.0	2.3	0.9	1.3	8.4
7	0.8	F	0.2	0.1	5.2	1.2	3.1	8.8	0.3	2.4	5.4
8	18.1	F	2.6	6.6	17.7	0.7	9.2	8.3	2.1	5.1	33.3
9	7.7	M	0.6	1.9	18.8	0.2	0.8	17.7	0.5	4.5	11.5
median	7.6	-	0.4	1.6	12.0	0.7	1.0	7.5	0.9	2.2	6.5

C)

ITP	fold increase caspase-3		fold increase caspase-9		fold increase caspase-8	
Patient #	Thrombin	A23187	Thrombin	A23187	Thrombin	A23187
1	1.3	1.6	1.1	1.1	1.9	1.4
2	1.6	4.7	1.9	1.7	1.9	2.2
3	2.1	4.3	3.5	2.4	1.1	1.7
4	1.3	2.8	0.9	2.1	1.7	2.4
5	0.4	0.8	0.1	0.5	0.4	0.7
6	2.3	1.9	4.5	2.9	2.5	2.1
7	2.7	2.9	1.0	9.2	0.9	1.2
8	2.7	2.3	2.2	1.4	1.3	0.5
9	0.9	2.5	5.5	8.6	3.8	4.9
10	3.3	5.7	1.2	2.1	1.0	4.1
median	1.8	2.7	1.6	2.1	1.5	1.9

D)

control	fold increase caspase-3		fold increase caspase-9		fold increase caspase-8	
Patient #	Thrombin	A23187	Thrombin	A23187	Thrombin	A23187
1	7.9	32.9	1.6	14.5	1.4	10.9
2	10.0	54.3	4.3	29.5	0.9	5.3
3	2.6	4.2	1.5	3.2	2.1	4.6
4	7.1	40.1	2.3	5.9	1.5	4.5
5	2.6	34.0	0.1	3.5	0.8	3.9
6	2.6	60.5	2.4	5.8	1.4	8.9
7	0.6	30.6	2.6	7.3	8.8	19.9
8	2.5	6.7	13.7	12.4	2.4	15.9
9	3.3	31.9	4.7	104.1	9.2	23.5
median	2.6	32.9	2.4	7.3	1.5	8.9

Table 3.

Platelet activation in acute ITP patients compared to control as determined by activated caspases. Analysis of activated caspase-3, caspase-8 and caspase-9 in platelets of resting platelets, platelets stimulated with thrombin and platelets stimulated with A23187 in acute ITP patients at diagnosis **(A)** and in control **(B)** by FAM-DEVD-FMK, FAM-LETD-FMK or FAM-LEHD-FMK fluorescence. The fold increase of the proportions of platelets showing active caspases was analyzed by comparing thrombin- and A23187- stimulated platelets to resting platelets, respectively, in ITP patients **(C)** and in healthy control children **(D)**. $p < 0.01$ for activated caspase-3, -9 and -8 between ITP and control in resting platelets. $p < 0.01$ for activated caspase-3, -9 and -8 between ITP and control in thrombin-stimulated platelets. $p < 0.05$ for activated caspase-9 and -8 between ITP and control in A23187-stimulated platelets.

Discussion

In this study we investigated markers for platelet activation as well as the generation of the endogenous thrombin potential (ETP) in a clinically well-defined group of children with very low platelet counts that presented with bleeding symptoms including petechiae, large hematomas, mucosal bleeding and epistaxis all typical sings of primary ITP. We compared these results in ITP to those from healthy children and children with chemotherapy-related thrombocytopenia.

We demonstrated that platelets of children with ITP have an increased CD62P as well as CD63 surface expression compared to both healthy and cTP children (Figure 1A & B). We further have shown that thrombin-induced activation in platelets of ITP patients is reduced similar to that in cTP (Figure 1A – C). The reduced thrombin-induced binding of PAC-1 might be responsible for the bleeding signs observed in ITP as binding of fibrinogen to activated GPIIb/IIIa is a final step in platelet aggregation. We also detected a reduced ETP in acute ITP patients as well as in chronic ITP patients but not in cTP patients (Figure 2). Furthermore, we indicated that in ITP patient's platelets caspase-3 activation is increased by thrombin- as well as by A23187 (Table 3) while CD63 and CD62P activation in response to thrombin is impaired (Figure 1). Thus we assume that thrombin signaling in ITP patient's platelets is functional.

After IVIg treatment, the bleeding symptoms of ITP subsided and thrombin-induced platelet activation was ameliorated as well as ETP. Following IVIg treatment, plasma of ITP patients presented a similar ETP than healthy children even if the platelet count in ITP after treatment was not normalized (Figure 2). Although ETP correlated to the platelet count, the effect of IVIg on ETP can not be explained by a rise in platelet count alone. After IVIg treatment, ITP patients show less bleeding signs without presenting a normal platelet count as seen in controls. This is confirmed by the fact that patients suffering from a thrombocytopenia due to chemotherapy have a normal ETP despite their low platelet count. Thereby we confirm that platelets are not required for the generation of thrombin. Severe bleeding only occurs at a very low platelet count in acute patients and less in chronic patients. Treatment with IVIg ameliorated most abnormal signs observed in ITP patients at diagnosis as platelet activation, apoptosis and ETP. Thus we conclude that IVIg is an efficient treatment in ITP patients and its high cost is justified by its efficacy and by the patient's safety. So far, next to the platelet count no other biological markers have been correlated to bleeding symptoms in ITP [21]. It will be necessary to include more subjects in the measurements of ETP to evaluate whether ETP is a potential biomarker for estimating the bleeding risk in thrombocytopenic patients.

In this study we also wanted to identify whether apoptosis correlates to platelet activation. It has been demonstrated that platelet activation and platelet apoptosis are different phenomena [22]. Here we show that platelet activation occurs in the same patients as are included in the study of platelet apoptosis in ITP [13]. We detected increased platelet activation in acute ITP in support with studies in chronic ITP [15] [16] [23]. We also show a reduced thrombin generation that might be responsible for reduced aggregation, thus bleeding. Another study demonstrated that sera from ITP patients either impair or enhance platelet aggregation [6]. Various other diseases have been associated with platelet dysfunction, activation or apoptosis as the Bernard-Soulier syndrome (BSS). BSS is an inherited platelet function disorder resulting in macro thrombocytopenia and bleeding. Patients present with low thrombin concentrations as well as increased CD62P and apoptotic events in platelets [24]. Despite the fact that ITP patients at diagnosis present increased CD62P and CD63 expression we did not detect a correlation of these activation markers to caspase activation. These data agree with the finding that PS exposure or microparticles are responsible for platelet activation and not expressed CD63 and CD62P. Furthermore, expression of CD63 and CD62P is crucial for an interaction of platelets with binding partners of the endothelial surface.

In Table 3 we demonstrated that activated caspase-3 in resting platelets of ITP patients can be activated by thrombin similar to resting control platelets. In contrast, CD63 and CD62P stimulation by thrombin was impaired. This finding indicates that thrombin stimulation is not entirely impaired in platelets of ITP patients. In contrast to caspase activation by thrombin, caspase activation by A23187 is significantly decreased in ITP platelets compared to control. The observed reduced fold increase of A23187-stimulated ITP platelets might be a consequence of a disruption in the Ca^{2+} homeostasis and an increase in the intracellular Ca^{2+} level. However, why thrombin stimulation is impaired in ITP platelets regarding CD63 and CD62P expression remains to be elucidated. We hypothesize that interfering autoantibodies on the platelet surface might be involved in the impaired activation of platelet surface proteins and are responsible for the observed increase in caspases.

Furthermore, we observed that caspase-8 is activated by thrombin as well as by A23187 in control or ITP platelets (Table 3). Caspase-8 is also activated in resting ITP platelets as we already have demonstrated earlier in the study of Chapter 2. These data confirm that the intrinsic and extrinsic apoptotic signaling pathway lead to an activation of caspases in platelets of ITP at diagnosis and also in the stimulation of platelets by agonists. However, a stimulation of platelets by A23187 should only result in an increase of caspase-3 and -9 activation as a disruption in the Ca^{2+} homeostasis initiates the intrinsic pathway via loss of the mitochondrial membrane potential. We therefore suggest that a positive feedback signaling is responsible for activation of caspase-8 in response to activated caspase-3. In

future studies, we aim to determine changes in the intracellular Ca^{2+} level in platelets of ITP patients by the fluorescent Ca^{2+} indicator Fura-2. We also want to determine whether caspase-8 is inhibited by the inhibitor of apoptosis cIAP-1. We consider inhibiting cIAP-1 by an artificial compound in a similar way as antagonizing XIAP with its inhibitor embelin as described in the study of Chapter 4. Furthermore, preliminary data indicate that platelets of chronic ITP patients have less activated caspases than of acute ITP underlying the assumption that etiology in chronic ITP is different from acute ITP. However, more chronic patients need to be investigated before defining a conclusion.

To conclude, our results show that platelets of ITP patients at diagnosis are pre-activated and that the capacity for further activation of these platelets on thrombin stimulation is impaired. However, GPIIb/IIIa was not activated in ITP platelets at diagnosis indicating that ITP platelets are not fully pre-activated. Besides, ITP patients have a low ETP. Treatment with IVIg ameliorates both thrombin activation and ETP in pediatric ITP patients. However, pre-activation of CD62P and CD63 are not significantly ameliorated by IVIg but a tendency is observed.

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Authorship and Disclosures

OS, JW, MS: designed the study; JW, OS: performed the experiments, JW performed all experiments except the experiment of Figure 2 (performed by AF) and parts of experiments of Figure 1 (performed by OS); JW, OS: analyzed data; SK, MS: attended and enrolled the patients and collected clinical data; and JW, OS, SK: wrote the manuscript. The authors have no conflicts of interest to declare.

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CHAPTER 4

Original Article
Platelets

Omi/HtrA2 and XIAP are components of platelet apoptosis signaling

Jeannine Winkler[1,2], Margaret Rand[3], Markus Schmugge[1], Oliver Speer[1,2],

[1]*Division of Hematology and Children's Research Center, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland*
[2]*Zurich Center for Integrative Human Physiology, University of Zurich, Switzerland, University of Zurich, Switzerland*
[3]*Division of Hematology / Oncology, The Hospital for Sick Children, 555 University Ave, Toronto, ON M5G 1X8, Canada*

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Correspondence: Dr. Oliver Speer, Division of Hematology, University Children's Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland
E.Mail: oliver.speer@kispi.uzh.ch

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Summary

Although platelets possess the hallmarks for apoptosis as activation of caspases, cytochrome c release and depolarization of the mitochondrial transmembrane potential ($\Delta\psi M$), their apoptotic signaling pathway is not entirely understood. In this study expression as well as inhibition of Omi/HtrA2 by Ucf-101 and of XIAP by embelin were investigated by Western blot and flow cytometry in human platelets with and without apoptotic stimuli. We found that platelets contain the pro-apoptotic protease Omi/HtrA2, the pro-apoptotic protein Smac/Diablo and the X-linked inhibitor of apoptosis XIAP. Omi/HtrA2 and Smac/Diablo were released from mitochondria into the cytosol together with cytochrome c after induction of apoptosis by the Ca^{2+} ionophore A23187 or the BH3 mimetic ABT-737. Exposure of platelets to Ucf-101 did neither abolish loss of $\Delta\psi M$ nor impair activation of caspase-8, however, Ucf-101 prevented apoptosis observed by a decrease in activated caspase-3 and caspase-9. In contrast, embelin promoted apoptotic events such as caspase-3 and -9 activation. These results verify that platelets have a complete intrinsic apoptotic signaling pathway including the pro-apoptotic protease Omi/HtrA2 and its target protein XIAP suggesting that platelet survival might be regulated by these proteins in addition to Bcl-family members.

Keywords: apoptosis, caspase-3, platelets, Omi/HtrA2, XIAP

Introduction

Apoptosis in nucleated cells is a well-known process regulating cell life span and the elimination of damaged or infected nucleated cells. Two different pathways of apoptosis have been described: the extrinsic pathway, initiated by death receptors; and the intrinsic pathway, initiated by stress signals e.g. DNA damage or disruption in the Ca^{2+} homeostasis. In addition, the extrinsic and intrinsic pathway can be connected by the BH3-interacting domain death agonist Bid that, once cleaved by caspase-8, translocates to the mitochondria and initiates oligomerization of Bax leading to mitochondrial outer membrane permeabilization (MOMP) [1] [2, 3]. Disruption of the Ca^{2+} homeostasis can also lead to MOMP, resulting in release of cytochrome c, Smac/Diablo, and the serine protease Omi/HtrA2 from their mitochondrial intermembrane space (IMS). Cytochrome c, together with Apaf-1 and dATP, forms the apoptosome, which then activates the initiator caspase-9. This initiator caspase then activates the effector caspases, caspase-3 and caspase-7 [1].

Additionally, caspases are negatively regulated by inhibitors of apoptosis (IAP) such as cIAP or XIAP. XIAP is known to bind and inhibit caspase-3, -7 and -9 [4-6]. This is achieved via the characteristic protein domains of IAPs, the so-called baculovirus inhibitor of apoptosis protein repeats, or BIR motifs [7, 8]. The mitochondrial BIR3-binding protein, Omi (also known as HtrA2), that is released from the IMS into the cytosol upon MOMP, was identified as a caspase activator [6, 9, 10]. In addition, Omi/HtrA2 was shown to induce apoptosis in human cells in a caspase-independent manner through its protease activity, and in a caspase-dependent manner via its ability to disrupt caspase-IAP interaction [9]. Knockdown of Omi/HtrA2 increases the resistance of multiple cell lines to apoptotic stimuli, such as staurosporine, cisplatin, UV irradiation, anti-Fas and TRAIL [5, 9, 11, 12]. Moreover, the synthetic Omi/HtrA2 inhibitor Ucf-101 [13], partially protects from cell death induced by cisplatin, [14] or TNF-α [15]. Similar to Omi/HtrA2, Smac/Diablo promotes caspase activation and apoptosis by binding to the BIR3 and BIR2 domains of XIAP and disrupting their interaction with caspase-9 and the effector caspases-3 and -7 [10, 16, 17].

Although platelets are anucleate, they undergo apoptotic-like events, including MOMP with collapse of the mitochondrial inner membrane potential ($\Delta\Psi m$) and release of cytochrome c into the cytoplasm [18]. After platelet stimulation with thrombin, Lopez et al. reported truncation and translocation of Bid, a pro-apoptotic Bcl-2 protein, to mitochondria, inducing Bak and Bax oligomerization followed by cytochrome c release [19]. It is well accepted that the anti-apoptotic Bcl-XL counteracts Bax and protects mitochondria in platelets [20, 21]. Once Bcl-XL becomes inhibited, Bax oligomerizes and forms pores in the outer mitochondrial membrane releasing cytochrome c from the IMS [22, 23]. However, it is not yet known whether, in platelets, Omi/HtrA2 and Smac/Diablo are released from

mitochondria. However, in platelets, release of cytochrome c [24] and activated Caspase-3 [23, 25] have been observed. In addition to these signs of apoptosis, increased microparticle formation [26], increased phosphatidylserine (PS) exposure, but also decreased life span of platelets leading to thrombocytopenia [20, 21] have been reported. Thus, these apoptotic events observed in platelets appear to be relevant in thrombocytopenia [27, 28]. Additionally, apoptotic events have been reported to occur during platelet storage [29] or after treatment with chemotherapeutic agents [20, 21, 23, 30].

There is a growing body of evidence that apoptotic events in platelets are controlled both by anti- and pro-apoptotic proteins [18, 21, 23]. Thus, we were interested in determining whether platelets, in addition to cytochrome c, might also release the mitochondrial proteins Omi/HtrA2 and Smac/Diablo from the IMS into the cytosol, activating caspases by antagonizing IAPs. We showed that Omi/HtrA2, Smac/Diablo and XIAP are expressed in human platelets. Using Ucf-101, an Omi/HtrA2-inhibitor previously studied in cell lines [13], and embelin, a XIAP inhibitor [31, 32], we demonstrated that inhibition of Omi/HtrA2 prevents apoptosis in human platelets, whereas inhibition of XIAP by embelin promotes platelet apoptosis.

Materials and methods

Materials

Collagen (200 µg/mL) was obtained from HART Biologicals (Hartlepool, England); thrombin, A23187, PGE_1, PGI_2, GPRP, digitonin, BSA and embelin were from Sigma-Aldrich (Buchs, Switzerland); PPACK and the protease inhibitor 5-[5-(2-nitrophenyl) furfuryliodine]-1,3-diphenyl-2-thiobarbituric acid (Ucf-101) were from Merck Chemicals (Zug, Switzerland); and ABT-737 was from Active Biochemicals (Maplewood, NJ, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (Buchs, Switzerland).

For Western blotting, anti-human caspase-3 mouse monoclonal antibody (mAb) (clone 3G2), anti-human caspase-7 mouse mAb (clone C7), anti-human caspase-9 rabbit polyclonal antibody (pAb), anti-human Omi/HtrA2 rabbit pAb, anti-human rabbit gelsolin pAb and anti-human β-actin mouse mAb (clone AC-74) were from Cell Signaling Technology (Boston, MA, USA) and anti-human rabbit mAb cleaved caspase-3 (clone 269518), anti-human Apaf-1 mouse mAb (clone 94408), anti-human cytochrome c mouse pAb (clone 7H8.2C12), anti-human Smac/Diablo rabbit pAb and anti-human XIAP mouse mAb (clone 117320) were from R&D Systems (Minneapolis, MN, USA). The caspase-3 antibody was used to detect the pro-caspase-3 (full length of 30kDa) and the cleaved caspase-3 antibody was used to detect the cleaved, active 17kDa fragment.

For flow cytometry, PerCP-conjugated anti-CD42a (glycoprotein (GP) IX; clone BEB1), APC-conjugated anti-CD62P (clone AK-4), PE-conjugated anti-CD63 (clone H5C6), as well as APC-labelled annexin A5 (that binds to cell-surface exposed phosphatidylserine (PS)) were used. Labelled mAbs and annexin A5 were purchased from Becton and Dickinson (Rotkreuz, Switzerland); fluorochrome inhibitors of caspases (FLICA) FAM-DEVD-FMK specific for active caspase-3, FAM-LETD-FMK specific for active caspase-8 and FAM-LEHD-FMK specific for active caspase-9 were from Millipore (Zug, Switzerland); the voltage-dependent, membrane intercalating dye tetramethylrhodamine-ethyl-ester (TMRE), used to analyze the mitochondrial inner membrane potential ($\Delta\Psi m$), was purchased from Invitrogen (Basel, Switzerland). 0.9 µm polystyrene latex marker beads used to discriminate platelets from platelet-derived microparticles were purchased from BioCytex (Marseille, France).

Blood sampling, preparation of washed platelets and platelet activation

This study was approved by the local ethics committee and written informed consent was obtained from the blood donors. Venous blood samples from healthy donors who had not ingested any anti-platelet medications for at least two weeks were collected into citrate anticoagulant (final concentration, 10.5 mM). Preparation of suspensions of washed platelets and platelet activation were carried out as described previously [33]. Briefly,

platelet-rich plasma (PRP) was obtained by centrifugation at 140g for 10 min at 22°C without braking, was removed to another tube and was supplemented with ACD (85 mM trisodium citrate, 78 mM citric acid, 110 mM glucose). PRP was centrifuged at 1200g for 12 min. The resulting platelet pellet was resuspended in Ca^{2+}-free Tyrode-Hepes buffer (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose and 5 mM HEPES) containing 0.02% EGTA and 0.35% albumin, pH 6.5. After each resuspension step, platelets were left at 22°C for 10 min and complemented with 72 µl ACD per mL of wash solution before centrifugation at 800g. The second washing medium was Ca^{2+}-free Tyrode-Hepes buffer without EGTA and the final resuspension medium was Tyrode-Hepes solution (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, and 5 mM HEPES, pH 7.4) supplemented with 0.35 % BSA (TAH), 0.05 U/mL apyrase and 2.5 mM GPRP. The platelet count was adjusted to 1 x 10^9/mL. Platelets were activated with either 10 µg/mL collagen in combination with 1 U/mL thrombin for 5 min or with 3 µM Ca^{2+} ionophore A23187 for 10 min. Platelet apoptosis was induced by 3 µM ABT-737 for 2 hr at 37°C. PGE_1 as well as PPACK were used to stop the activation of collagen and thrombin stimulated platelets as previously described [33].

To investigate the effect of blocking Omi/HtrA2, platelets were pretreated with 10 µM Ucf-101 for 2 hr as described in mouse fibroblasts [14] at 37°C before addition of agonist(s) or ABT-737. To investigate the effect of blocking XIAP, platelets were pretreated with 20 µM embelin for 2, 22 hr or 48 hr as described in prostate cancer or leukemic cells [32] at 37°C

Western blotting

For preparation of cell lysates, a platelet pellet obtained by centrifugation of washed platelets incubated with 0.5 mM PGI_2 at 500g for 10 min was lysed in lysis buffer (50 mM TrisHCl, 150 mM NaCl, 1% Tween, 1 mM EDTA, pH 7.4). The platelet lysates were cleared by centrifugation at 4°C and protein concentrations were determined. Alternatively, the release of mitochondrial proteins into the cytoplasm of platelets was assessed by lysis of platelet pellets in mitochondrial isolation buffer (250 mM sucrose, 20 mM Hepes pH 7.4, 5 mM $MgCl_2$, 10 mM KCl, 0.05% digitonin) following differential centrifugation as described by Vogler et al [23]. At this concentration, digitonin mainly permeabilizes the platelet plasma membrane. Thus, after centrifugation, the supernatant (SN) contains soluble cytosolic proteins and also any proteins released from mitochondria while the platelet pellet contains heavy protein complexes and membranes (HM) including mitochondria and the platelet cytoskeleton. Equal amounts of protein were electrophoretically separated on 4-12% BisTris NuPage gels (Invitrogen, Basel, Switzerland), and transferred to nitrocellulose membranes (Bio-Rad Laboratories AG, Reinach, Switzerland) that were blocked with 5% non-fat dried milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.4) with 0.1% Tween-20.

Membranes were then incubated with primary antibodies followed by HRP-conjugated secondary antibodies (Jackson Immunology, Newmarket, UK) and developed with ECL reagent (GE Healthcare, Glattbrugg, Switzerland) or Visualizer Western Blot detection kit (Millipore, Zug, Switzerland) and autoradiography. Membranes were stripped (1% SDS, 25 mM glycine, 2 mM dithiothreitol, pH 2) once or twice for reprobing.

Flow cytometry

Flow cytometric analyses were done using washed platelets. To analyze markers of platelet activation, washed platelets were diluted 50-fold with Tyrode-Hepes solution (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, and 5 mM HEPES, pH 7.4) supplemented with 0.35 % BSA (TAH) , containing anti-CD42a-PerCP (used as the platelet marker), anti-CD63-PE and anti-CD62P-APC (used as markers for platelet activation). To analyze markers of platelet apoptosis, washed platelets were incubated under the same conditions with either anti-CD42a-PerCP (used as the platelet marker), annexin A5-APC (to detect PS exposure) and FAM-DEVD-FMK-FITC (to determine caspase-3); anti-CD42a-PerCP (used as the platelet marker) and FAM-LETD-FMK-FITC (to determine caspase-8); or anti-CD42a-PerCP (used as the platelet marker) and FAM-LEHD-FMK-FITC (to determine caspase-9). For the measurement of $\Delta\Psi m$, platelets were incubated with the platelet marker anti-CD42a-PerCP and 100 nM TMRE. After 1 hr incubation at 22°C, samples were diluted further 10-fold with Tyrode-Hepes lacking BSA (TH). For each sample, 10'000 platelets, identified as CD42a-positive events, were acquired using a FACSCalibur flow cytometer (Becton Dickinson, Rotkreuz, Switzerland). Results are expressed as the percentage of platelets binding FAM-DEVD-FMK, FAM-LETD-FMK, FAM-LEHD-FMK, annexin A5, or anti-CD62P and anti-CD63 antibodies. For $\Delta\Psi m$ analysis, the geometric mean fluorescence intensity (MFI) was quantified. The percentage of platelet-derived microparticles was defined as the proportion of CD42a-positive events < 0.9 µm determined using calibrated beads as described [34]. Data were analyzed using FCS Express (De Novo Software, Los Angeles, CA, USA).

Statistical analysis

All data are expressed as mean ± SEM, where n = the number of independent experiments performed with different blood donors. Statistical analysis was done using Kruskal-Wallis one-way ANOVA, followed by the Mann Whitney test (Figure 3 & 5) and paired t-test (Figure 4 & 6). $p < 0.05$ was considered statistically significant. Analysis was done using SPSS (Dynelytics, Zurich, Switzerland).

Results

We first systematically investigated the expression of pro- and anti-apoptotic proteins of the intrinsic pathway in human platelets; by Western blot analysis, we detected the pro-apoptotic proteins Omi/HtrA2, Smac/Diablo, cytochrome c, Apaf-1, caspase-9, caspase-3 and caspase-7 and the anti-apoptotic protein XIAP in washed platelets (Figure 1). Caspase-3 presented as a double band in some donors. We also detected the pro-apoptotic proteins Bid, Bax and Bad as well as the anti-apoptotic protein Bcl-XL (data not shown). These data confirm the presence of Bid, Bax, Bad, Bcl-XL, cytochrome c, Smac/Diablo and Apaf-1 that have been reported previously in platelets [24, 35]; however to our knowledge neither the pro-apoptotic Omi/HtrA2 nor its target anti-apoptotic protein XIAP have been reported to be expressed in human platelets.

Increases in platelet cytosolic Ca^{2+} induced by Ca^{2+} ionophore A23187 and platelet apoptosis induced by ABT-737 lead to Omi/HtrA2, Smac/Diablo and cytochrome c release from mitochondria into the cytosol.

To elucidate whether during apoptotic signaling in platelets Omi/HtrA2 and Smac/Diablo, in addition to cytochrome c [21], are released from mitochondria into the cytosol, washed platelets were treated with the combination of the physiological stimuli collagen and thrombin (C+T), the Ca^{2+} ionophore A23187, or the BH3-only mimetic ABT-737. In contrast to C+T, both A23187 and ABT-737 induced release of Omi/HtrA2, Smac/Diablo and cytochrome c from mitochondria (Figure 2A). In addition, we detected the activated forms of caspase-7 and caspase-3 in platelets stimulated with A23187 as well as with ABT-737 confirming that these stimuli induced apoptotic events (Figure 2B). The active forms of caspase-3 and -7 were also detected in C+T stimulated platelets (Figure 2B). As described in nucleated cells, the data in Figure 1 and Figure 2A & B demonstrate that anucleated platelets also contain a complete intrinsic apoptosis-signaling pathway including Smac/Diablo and Omi/HtrA2 that can be released from mitochondrial IMS into the platelet cytosol, as well as the cytosolic anti-apoptotic XIAP.

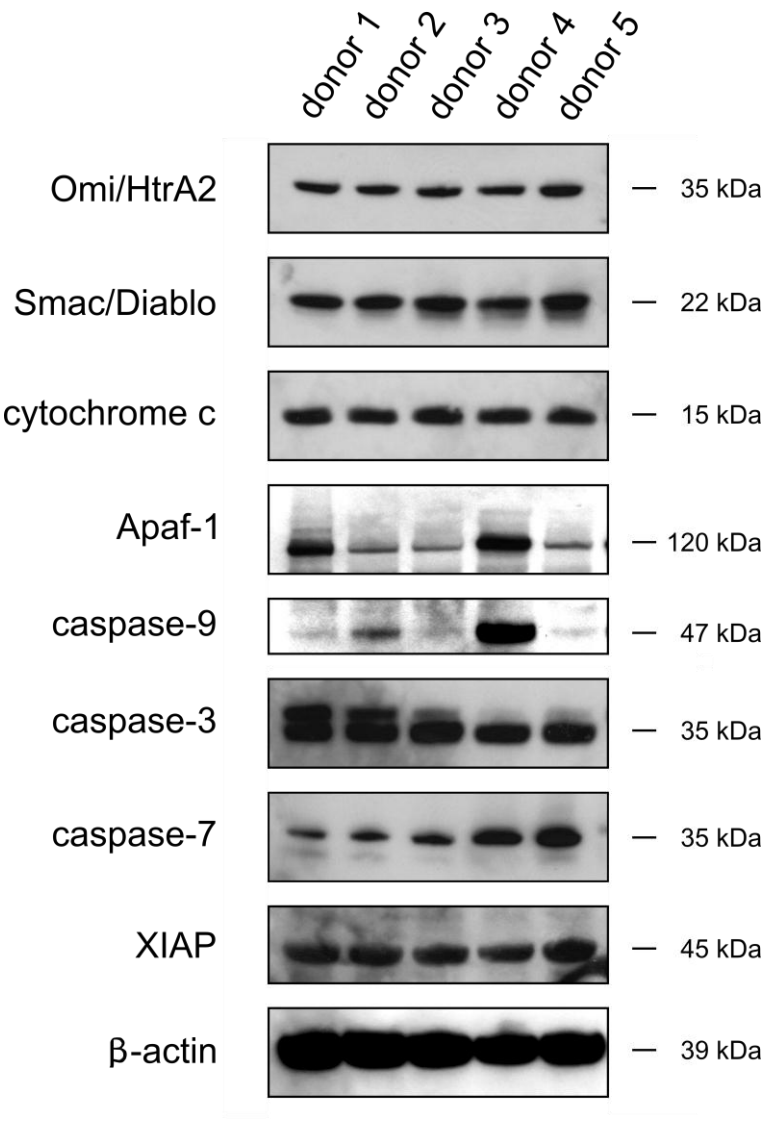

Figure 1. Platelets express the necessary components for a fully functional intrinsic apoptosis pathway. Western blot analysis of pro- and anti-apoptotic proteins of the intrinsic apoptosis pathway in washed platelets from 5 different donors. β-actin was included as loading control (representative of 3 experiments). Molecular weights are as indicated.

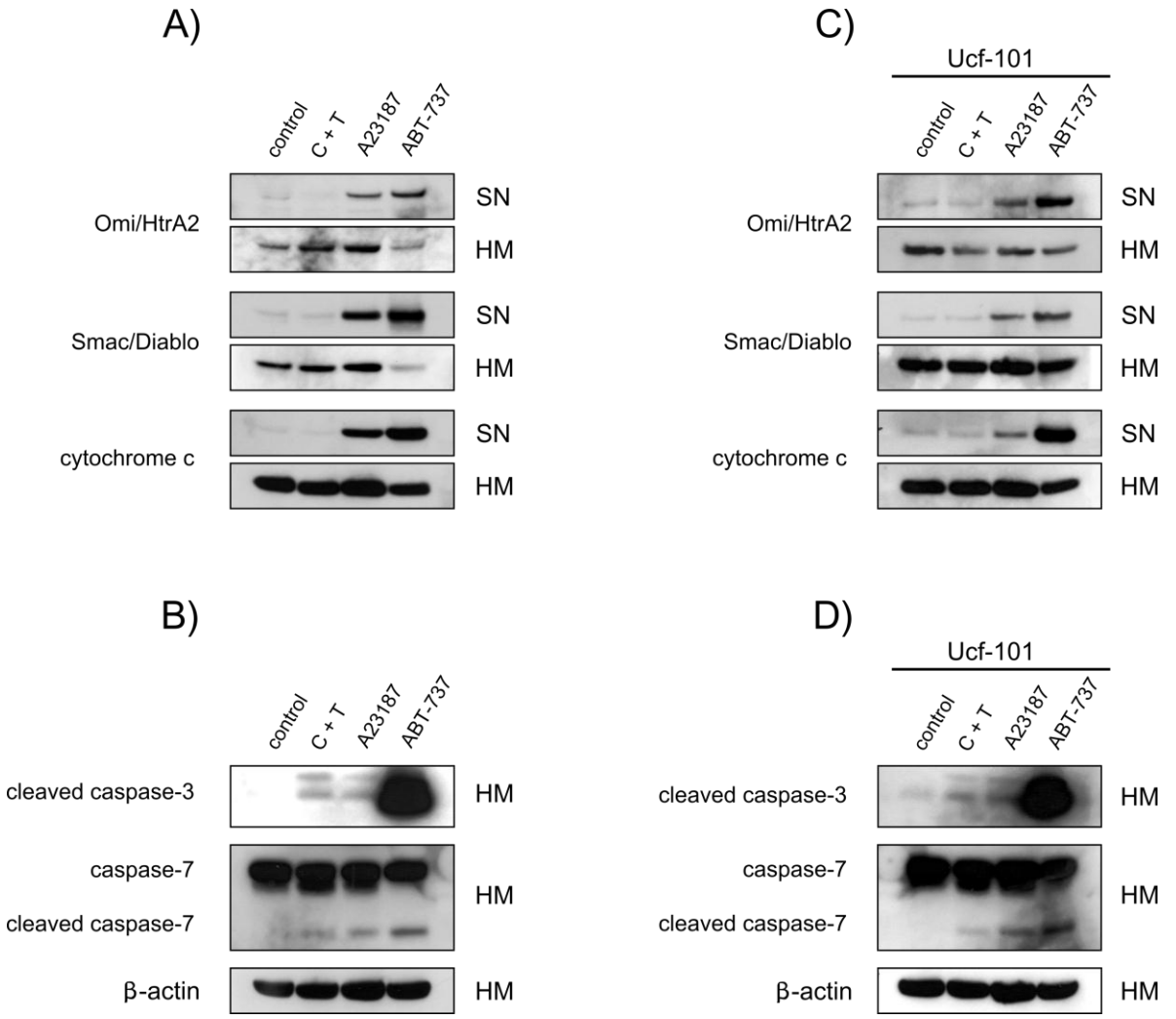

Figure 2. Release of mitochondrial intermembrane space (IMS) proteins from mitochondria into the cytoplasm after induction of apoptosis in control platelets (A) as well as in Ucf-101 treated platelets (C). Washed platelets (10^9/mL in Tyrodes-Hepes buffer) were stimulated with collagen (10 µg/mL) and thrombin (1 U/mL) (C + T) for 5 min, or Ca^{2+} ionophore A23187 (3 µM) for 10 min, or ABT-737 (3 µM) for 2 hr at 37°C. Release of mitochondrial IMS Omi/HtrA2, Smac/Diablo and cytochrome c was assessed by Western blot analysis of the supernatant (SN) containing soluble proteins that contain any proteins released from mitochondria and of the HM pellet containing heavy protein complexes and membranes including intact mitochondria and platelet cytoskeleton, isolated as described in Materials and Methods. Active caspase-3 and caspase-7 were analyzed in the HM **(B and D)**. Representative Western blots of 3 different donors for C+T and A23187 and of 2 different donors for control and ABT-737.

Inhibition of the pro-apoptotic protease Omi/HtrA2 with Ucf-101 diminishes activation of caspase-3 and -9

To investigate the role of Omi/HtrA2 during apoptotic signaling in platelets, we first characterized the impact of the Omi/HtrA2 inhibitor Ucf-101 on platelet function as determined by expression of platelet activation markers. Washed human platelets were incubated with increasing Ucf-101 concentrations (5 – 40 µM); up to a concentration of 40 µM, Ucf-101 pretreatment alone did not induce surface expression of CD62P (Figure 3A) or CD63 (Figure 3B). As well, pretreatment of platelets with Ucf-101 up to 10 µM did not affect expression of CD62P and CD63 in C+T-stimulated platelets (Figure 3A & B). Ucf-101 started to significantly inhibit platelet activation by C+T at 30 µM in regard to CD62P expression and at 20 µM in regard to CD63 expression. Furthermore, platelet aggregation in citrated-PRP in response to 2 mM arachidonic acid, 5 or 10 µM ADP or 10 µg/mL collagen was not impaired by 10 µM Ucf-101 (data not shown). Therefore, in further experiments, 10 µM Ucf-101 was used to examine the effects of this inhibitor on platelet apoptosis. To investigate whether Ucf-101 interferes with Omi/HtrA2-release from the mitochondrial IMS into the cytosol, washed human platelets were pretreated with Ucf-101 and stimulated with C+T or A23187 or with the apoptosis inducer ABT-737, and were analyzed for the release of mitochondrial IMS proteins into the cytosol. As in the absence of Ucf-101, pretreatment with 10 µM Ucf-10 induced a release of the IMS proteins Omi/HtrA2, Smac/Diablo and cytochrome c in A23187- and ABT-737 stimulated platelets into the cytosol (Figure 2C). We also detected the activated forms of caspase-7 and caspase-3 in platelets pretreated with Ucf-101 stimulated with A23187 as well as with ABT-737 confirming that these stimuli induced apoptotic events not only in untreated but also Ucf-101 treated platelets (Figure 2D). These data confirm that any inhibitory effect of Ucf-101 does not occur at the level of Omi/HtrA2 release into the cytosol from the IMS.

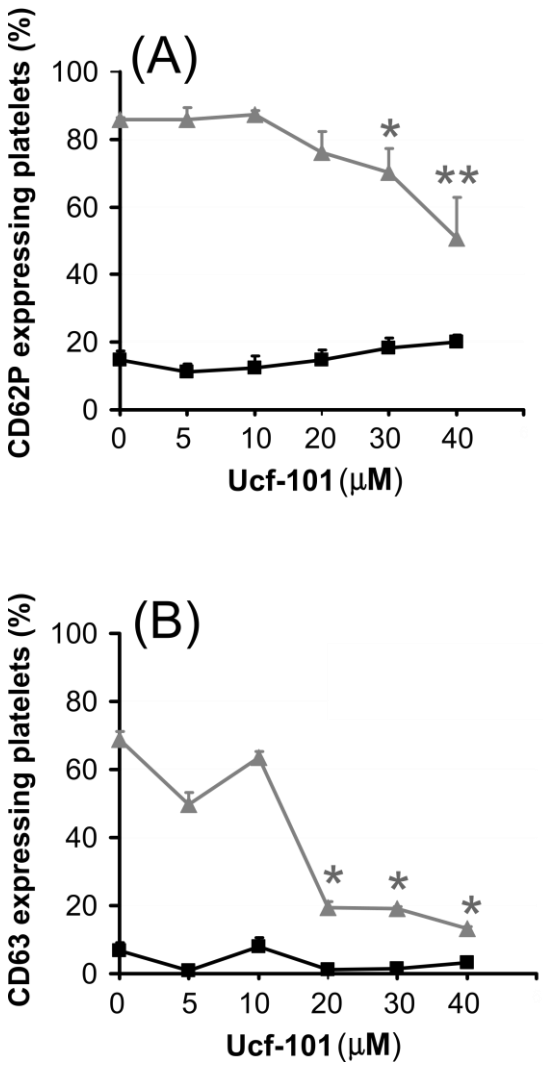

Figure 3. Effect of increasing Ucf-101 concentrations on platelet activation as determined by flow cytometry. Washed platelets (10^9/mL in Tyrode-Hepes buffer) were treated with 5 - 40 µM Ucf-101 for 2 hr at 37 °C and then stimulated with the combination of collagen (10 µg/mL) and thrombin (1 U/mL) (C + T) for 5 min at 37°C. The percentage of platelets expressing P-selectin, by APC-conjugated CD62P **(A)** and of platelets expressing CD63, by PE-conjugated CD63 **(B)**. Data are shown as mean ± SEM of at least 3 independent experiments (individual donors). Black squares: resting platelets treated with Ucf-101 alone; grey triangles: platelets pretreated with Ucf-101 and stimulated with C+T. * $p < 0.05$, ** $p < 0.01$ compared to C+T stimulated platelets pretreated with vehicle only (0 µM Ucf-101).

Next, washed human platelets were treated with C+T, the Ca^{2+} ionophore A23187 or the BH3 mimetic ABT-737 to induce apoptotic events in platelets: as determined by flow cytometry, C+T, A23187 and ABT-737 all induced statistically significant activation of caspase-3, caspase-8 and caspase-9 ($p < 0.01$ between ctrl and C+T and between ctrl and A23187; $p > 0.05$ between ctrl and ABT-737) (Figure 4 A-C). (Note that to determine whether only the intrinsic apoptotic pathway is activated or also the extrinsic, we investigated activation of caspase-8.) Furthermore, C+T, A23187 and ABT-737 induced formation of microparticles (Figure 4D), PS exposure (Figure 4E) and loss of the $\Delta\Psi m$ (Figure 4F). However, after pre-treatment with 10 µM Ucf-101, the proportion of platelets with activated caspase-3 decreased significantly (C+T: 3.7 ± 0.9%; A23187: 7.3 ± 1.1%; ABT-737: 9.7 ± 2%) (Figure 4A) compared to platelets not pretreated with Ucf-101 (C+T: 8.6 ± 1.2%; A23187: 19.8 ± 2.4%; ABT-737: 16.6 ± 2.5%) ($p < 0.5 - 0.1$). Ucf-101 pretreatment also decreased activation of caspase-9 (C+T: 2.5 ± 0.7%; A23187: 6.2 ± 1.9%; ABT-737: 11.8 ± 3.5%) compared to platelets in the absence of Ucf-101 (C+T: 6.9 ± 2.8%; A23187: 16.6 ± 4.7%; ABT-737: 17.9 ± 2.6%) (Figure 4B). In contrast, Ucf-101 pretreatment did not prevent caspase-8 activation or production of platelet-derived microparticles (Figure 4C & D). PS exposure was decreased in ABT-737 stimulated platelets pre-treated with Ucf-101 (77.2 ± 4.0%) compared to control (89.0 ± 1.1%) ($p < 0.05$) and loss of $\Delta\psi m$ was decreased in Ucf-101 pretreated platelets for C+T (MFI: 33.3 ± 5.1) compared to control (41.4 ± 4.0) as well as for A23187 (13.6 ± 3.5) compared to control (20.3 ± 7.4) (Figure 4E & F). Also, the inhibition of platelet activation by ABT-737 was not prevented by Ucf-101 (Figure 4G & H). Taken together, these data indicate that the Omi/HtrA2 inhibitor Ucf-101 inhibits activation of caspase-3 and caspase-9 in platelets.

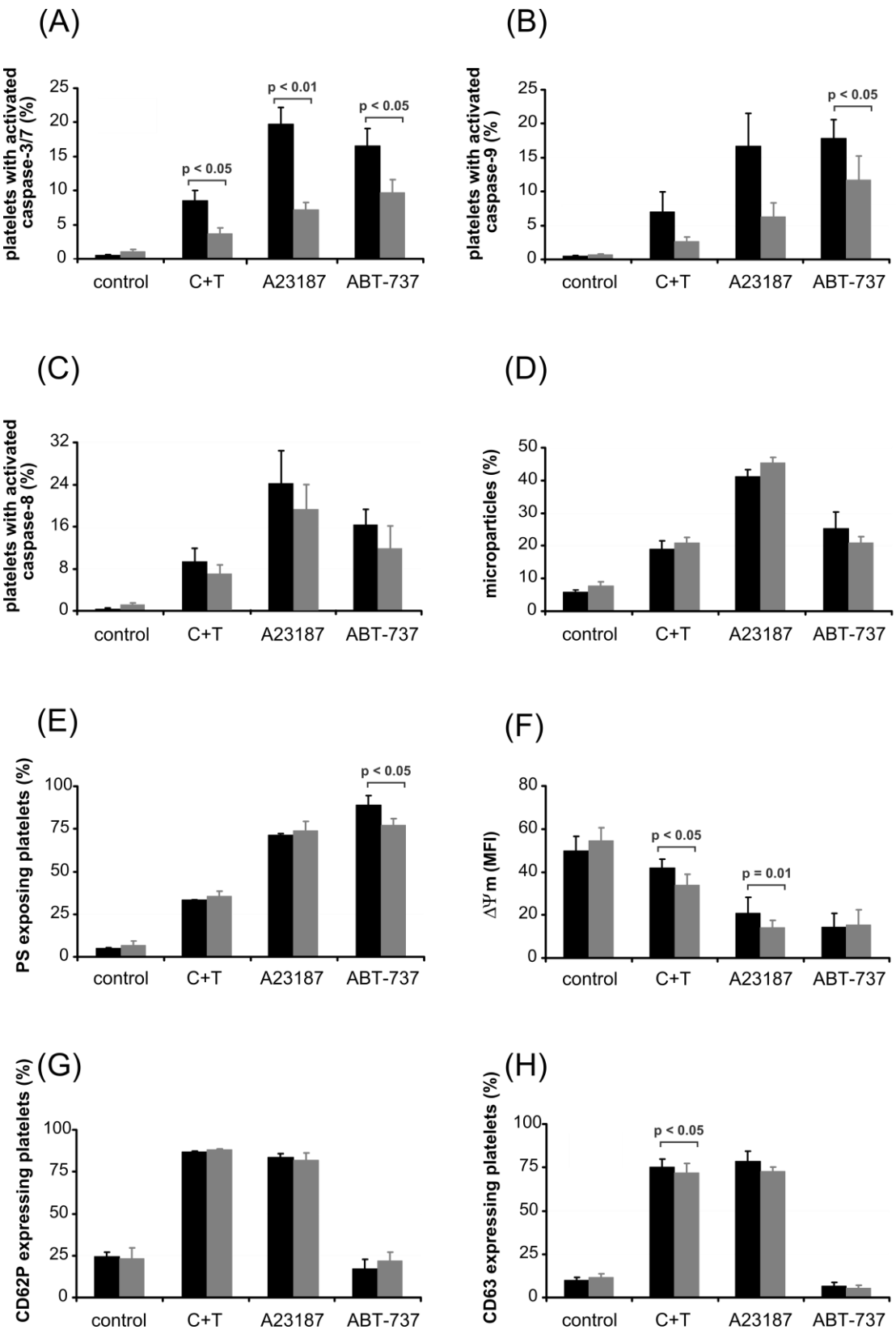
(A)
platelets with activated caspase-3/7 (%)
25
20
15
10
5
0
p < 0.05
p < 0.01
p < 0.05
control
C+T
A23187
ABT-737
(B)
platelets with activated caspase-9 (%)
25
20
15
10
5
0
p < 0.05
control
C+T
A23187
ABT-737
(C)
platelets with activated caspase-8 (%)
32
24
16
8
0
control
C+T
A23187
ABT-737
(D)
microparticles (%)
50
40
30
20
10
0
control
C+T
A23187
ABT-737
(E)
PS exposing platelets (%)
100
75
50
25
0
p < 0.05
control
C+T
A23187
ABT-737
(F)
ΔΨm (MFI)
80
60
40
20
0
p < 0.05
p = 0.01
control
C+T
A23187
ABT-737
(G)
CD62P expressing platelets (%)
100
75
50
25
0
control
C+T
A23187
ABT-737
(H)
CD63 expressing platelets (%)
100
75
50
25
0
p < 0.05
control
C+T
A23187
ABT-737

Figure 4. Decreased caspase-3 and caspase-9 activation in platelets treated with the Omi/HrA2 inhibitor Ucf-101, as analyzed by flow cytometry. Washed platelets (10^9/mL in Tyrode-Hepes buffer) were stimulated with 10 µg/ml collagen and 1 U/ml thrombin (C + T), 3 µM A23187 or 3 µM ABT-737 and compared to stimulated platelets that have been pretreated with 10 µM Ucf-101 for 2 hr at 37°C. The percentage of platelets with activated caspase-3 was determined by FAM-DEVD-FMK fluorescence **(A)**, of platelets with caspase-9 by FAM-LEHD-FMK fluorescence **(B)** and of platelets with activated caspase-8 by FAM-LETD-FMK **(C)**. The percentage of platelet-derived microparticles was determined as the proportion of CD42a-positive events <0.9 µm **(D)**. The percentage of platelets exposing PS was determined by annexin A5 binding **(E)** and $\Delta\Psi$m by mean fluorescence intensity (MFI) of TMRE (F). P-selectin expression was assessed by APC-conjugated anti-CD62P antibody **(G)** and CD63 expression, by PE-conjugated anti-CD63 antibody **(H)**. Black bars: platelets in the absence of Ucf-101; grey bars: platelets pretreated with Ucf-101. Data are shown as mean ± SEM of 5 experiments (individual donors), except for ABT-737 where 4 experiments were performed and $p < 0.05$ was considered as statistically significant.

Inhibition of platelet XIAP increases caspase activity

In nucleated cells, XIAP is an anti-apoptotic protein that inhibits active caspase-3 and caspase-9. The pro-apoptotic protein Omi/HtrA2 targets and inhibits XIAP. We continued our investigation examining caspase activation after the inhibition of XIAP by its inhibitor embelin that has previously been used in PC-3 prostate cancer cells or HL60 leukemic cells [31, 32, 36]. The pro-apoptotic effect of embelin is only observed after a long time course as described in leukemic cells [32] . Platelets incubated with 20 µM embelin for 22 hr did not present platelet activation as surface expression of CD62P (Figure 5A) or CD63 (Figure 5B), also after 48 hr, CD62P and CD63 expression were not statistically significantly elevated. To conclude, 20 µM embelin did not affect platelet activation. However, there was a tendency for increased CD63 expression after an incubation of 10 - 30 µM Ucf-101 for 48 hr and also for an increased CD62P expression even at 10 µM after 22 hr and after 48 hr. Incubation of platelets with 20 µM embelin led to an increase of activated caspase-3 and caspase-9 in up to 35% of platelets (Figure 6A & B). After incubation with embelin for 22 hr, the proportion of platelets with active caspase-3 was 4.1 ± 1.6% and of active caspase-9, 12.9 ± 7.2%, compared to a 2 hr embelin incubation with 0.3 ± 0.1% for active caspase-3 and 0.5 ± 0.2% for active caspase-9 that are similar to control levels at 22 hr (caspase-3: 0.7 ± 0.1%; caspase-9: 1.4 ± 0.8%). After a 48 hr incubation with embelin, values increased to 11.7 ± 2.9 % for active caspase-3 and to 19 ± 18.4% for active caspase-9 compared to control at 48 hr, in the absence of embelin (caspase-3: 1.0± 0.3%; caspase-9: 1.4 ± 0.4%). Caspase-8 levels were less increased (3.0 ± 2.4% after 22 hr and 9.9 ± 5.5% after 48 hr) (Figure 6C)

compared to caspase-3 and -9. Furthermore, a significant increase in the proportion of platelet-derived microparticles (Figure 6D) was observed. After an embelin incubation of 22 hr, microparticles increased to 10.4 ± 1.4% compared to control (6.2 ± 0.7%) and after 48 hr, values increased to 18.2 ± 1.7% compared to control (7.5 ± 0.8%). Platelets also presented significantly enhanced PS exposure with embelin incubation: 54.6 ± 10.4% after 22 hr compared to control 2.9 ± 0.8%, and 79 ± 18.2% after 48 hr compared to control 5.7 ± 18.2% (Figure 6E). Furthermore, treatment of platelets with embelin led to a loss of $\Delta\Psi m$ (Figure 6F), as reported by Hu et al. in leukemic cells [32]. After 22 hr platelets incubated with embelin presented a significantly decreased $\Delta\Psi m$ of 17.3 ± 2.0 compared to untreated platelets (51.4 ± 0.3) and after 48 hr of 15.8 ± 2.5 compared to untreated platelets (42.5 ± 0.2). The activation markers CD62P (Figure 6G) and CD63 (Figure 6H) were not significantly altered.

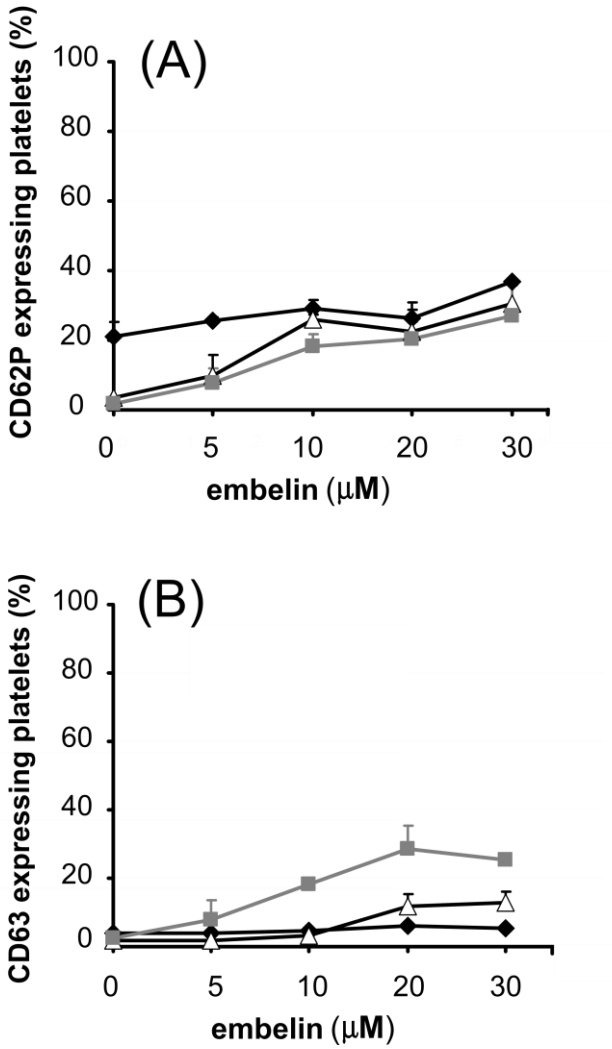

Figure 5. Effect of increasing embelin concentrations on platelet activation, as determined by flow cytometry. Washed platelets (10^9/mL in RPMI medium) were treated with embelin (5 – 30 µM) for 2, 22 and 48 hr at 37 °C under cell culture conditions. The percentage of platelets expressing P-selectin, by APC-conjugated CD62P **(A)**, and of platelets expressing CD63, by PE-conjugated CD63 **(B)**. Data are shown as mean ± SEM of at least 3 independent experiments (individual donors). Black diamonds: platelets treated with embelin for 2 hr; open triangles: platelets treated with embelin for 22 hr and grey squares: platelets treated with embelin for 48 hr.

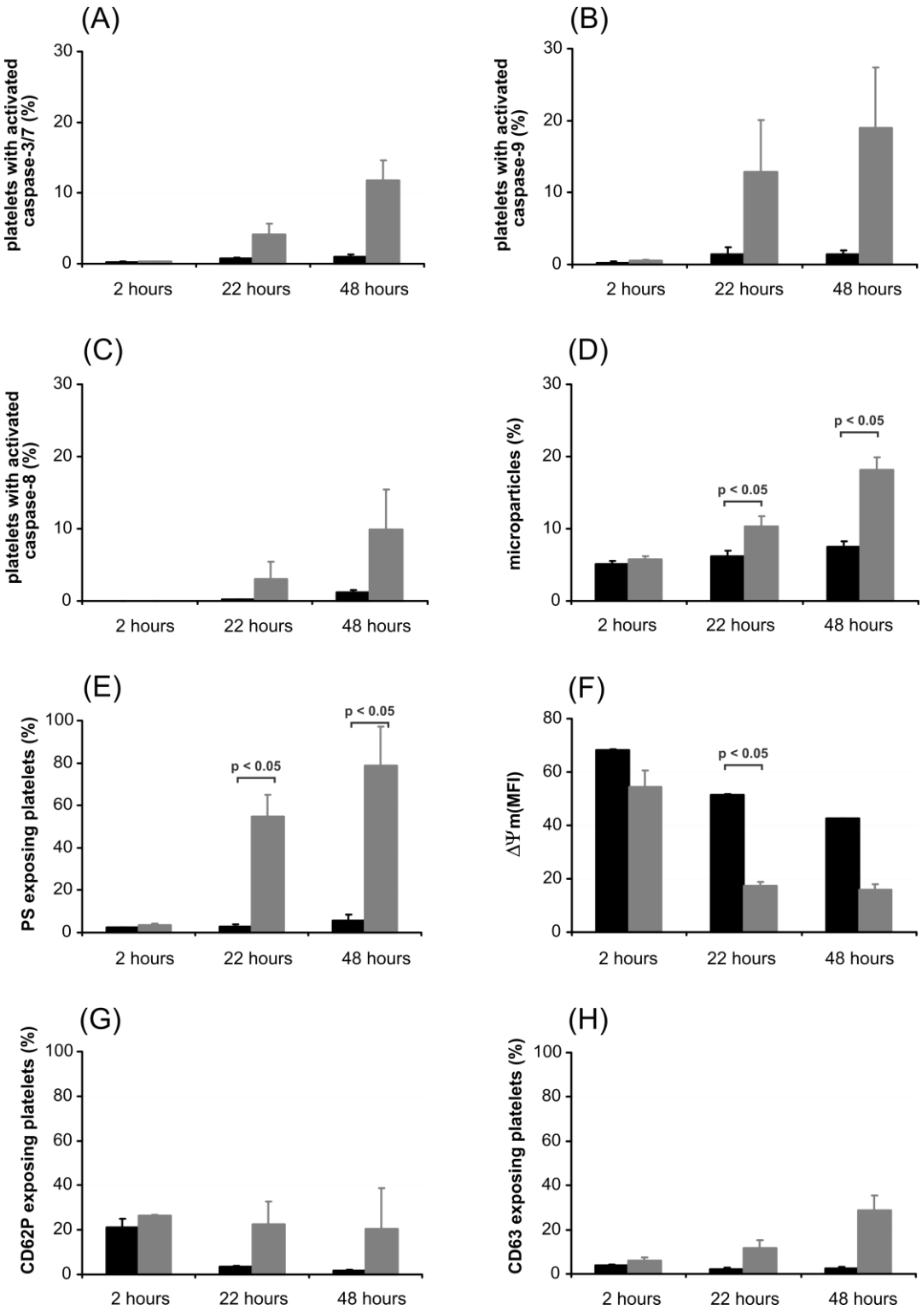
(A)
platelets with activated caspase-3/7 (%)
30
20
10
0
2 hours
22 hours
48 hours
(B)
platelets with activated caspase-9 (%)
30
20
10
0
2 hours
22 hours
48 hours
(C)
platelets with activated caspase-8 (%)
30
20
10
0
2 hours
22 hours
48 hours
(D)
microparticles (%)
p < 0.05
p < 0.05
30
20
10
0
2 hours
22 hours
48 hours
(E)
PS exposing platelets (%)
p < 0.05
p < 0.05
100
80
60
40
20
0
2 hours
22 hours
48 hours
(F)
ΔΨm(MFI)
p < 0.05
80
60
40
20
0
2 hours
22 hours
48 hours
(G)
CD62P exposing platelets (%)
100
80
60
40
20
0
2 hours
22 hours
48 hours
(H)
CD63 exposing platelets (%)
100
80
60
40
20
0
2 hours
22 hours
48 hours

Figure 6. Increased caspase-3 and caspase-9 activation in platelets treated with the XIAP inhibitor embelin, as analyzed by flow cytometry. Washed platelets (10^9/mL in RPMI medium) were incubated with 20 µM embelin for 2, 22 or 48 hr at 37°C under cell culture conditions. The percentage of platelets with activated caspase-3 was determined by FAM-DEVD-FMK fluorescence **(A)**, of platelets with caspase-9 by FAM-LEHD-FMK fluorescence **(B)** and of platelets with activated caspase-8 by FAM-LETD-FMK **(C).** The percentage of platelet-derived microparticles was determined as the proportion of CD42a-positive events <0.9 µm **(D)**. The percentage of platelets exposing PS was determined by annexin A5 binding **(E)** and the $\Delta\Psi$m by mean fluorescence intensity (MFI) of TMRE **(F)**. P-selectin expression was assessed by APC-conjugated anti-CD62P antibody **(G)** and CD63 expression, by PE-conjugated anti-CD63 antibody **(H)**. Black bars: platelets in the absence of embelin; grey bars: platelets treated with embelin. Data are shown as mean ± SEM of 3 experiments (individual donors), and $p < 0.05$ was considered as statistically significant.

Discussion

In the present study, we identified the pro-apoptotic protease Omi/HtrA2 and its target, the X-linked inhibitor of apoptosis (XIAP) as important components and regulators of apoptotic signaling pathways in platelets. Initially, we showed that the pro-apoptotic protease Omi/HtrA2 and the mitochondrial protein Smac/Diablo, as well as XIAP are expressed in platelets (Figure 1 & 2). Both Omi/HtrA2 and Smac/Diablo are released from mitochondria into the cytosol in parallel with cytochrome c after induction of apoptosis by disruption of platelet calcium homeostasis (with A23187 or ABT-737), leading to caspase-3 and -7 activation. By specifically inhibiting the pro-apoptotic protein Omi/HtrA2, the proportion of platelets showing active caspase-3/7 and -9 was diminished; however the proportion of platelets showing caspase-8 activation was not statistically decreased. This result supports our initial finding that platelets express inhibitor of apoptosis proteins such as XIAP that inhibits caspases-3/7 and -9. Additionally, direct inhibition of XIAP led to activation of caspase-3/7 also without disruption of calcium homeostasis by A23187 or targeting Bcl-XL and Bcl-2 by ABT-737. Of note is the fact that the physiological stimuli C+T did not induce release of Omi/HtrA2, Smac/Diablo and cytochrome c from mitochondria or only at the detection limit of Western blotting, thus caspase activation was only slightly increased.

Our findings support a number of previous reports: The expression of Apaf-1 and Smac/Diablo have previously been shown by Wolf et al. and Plenchette et al., respectively, in platelets [24, 35], and cytochrome c release and activation of caspase-9 and -3/7 after inhibition of Bcl-2 and Bcl-XL in platelets are well established to occur [22, 23, 25]. These platelet apoptotic events, especially caspase activation, appears to also play a role in settings of disease such as thrombocytopenia [28, 37], Omi/HtrA2 not only has XIAP as its substrate. Omi/HtrA2 was shown to degrade several proteins, preferentially cytoskeleton components [38]. This finding might explain the caspase independent effects of ABT-737 that were reported [23].

However, all stimuli that were used induced PS exposure and microparticle generation. On the basis of these results, we speculate that in resting platelets and in physiologically activated platelets, caspase activation is maintained at low levels by XIAP. Only if the mitochondrial integrity is disrupted, e.g. by ABT-737, A23187 or very likely by radical oxygen species as shown by Lopez et al. [22], cytochrome c, Omi/HtrA2 and Smac/Diablo are released from the mitochondrial IMS, leading to the formation of the apoptosome by Apaf-1, dATP and cytochrome c; and also to the degradation of XIAP by Omi/HtrA2 and Smac/Diablo, all culminating in the activation of caspase-9 and caspase-3/7. Activation of effector caspases leads to cleavage of target proteins such as PARP, gelsolin or α-fodrin [23, 25] including transmembrane proteins [39], therefore we can not exclude that

platelet glycoproteins are also targets for caspase-3/7 which might eventually explain the decrease in platelet activation after thrombin stimulation. However another likely cause for this observation might be the opsonization of the platelets by autoantibodies. Unfortunately so far we were not able study whether human anti human-platelet proteins lead to decreased platelet activability or even increased caspase activity.

We confirm that ABT-737 and the Ca^{2+} ionophore A23187, but less the combination of thrombin and collagen, can trigger platelet apoptosis [26, 40] leading to a depolarization of the mitochondrial transmembrane potential, phosphatidylserine exposure and activation of caspase-9, 3, -7, and -8 (Figure 4). We further show that Omi/HtrA2, Smac/Diablo and cytochrome c are released from mitochondria upon induction of apoptosis (Figure 2A) as previously indicated for cytochrome c [23], all in analogy to cytoplasmic apoptosis-events in nucleated cells [1]. Cilenti et al. showed that Ucf-101 functions as an Omi/HtrA2 inhibitor in mouse fibroblasts [13]. We demonstrate now that Ucf-101 inhibits apoptosis by reducing the activation of caspase-3 and caspase-9 but not of caspase-8 (Figure 4) suggesting that Ucf-101 binds the pro-apoptotic protein serine protease Omi/HtrA2. We then show that Omi/HtrA2 is released from the mitochondria in apoptotic platelets pre-treated with Ucf-101 (Figure 2C). This result indicates that the interaction of Omi/HtrA2 with Ucf-101 occurs in the cytosol and is a downstream effect of the MOMP. This finding is also confirmed by the fact that Ucf-101 does not abolish loss of the mitochondrial inner transmembrane potential in apoptotic platelets. It further confirms that Omi/HtrA2 is acting upstream of caspase-9 and caspase-3 and downstream of caspase-8. PS exposure however, has been reported to be a downstream manifestation of the intrinsic apoptotic pathway [41] but was not abolished by Ucf-101 in our study (Figure 4E). The proportion of platelet-derived microparticles was not decreased by Ucf-101 either in apoptotic platelets (Figure 4D). These data strongly suggest that in apoptotic platelets, Ucf-101 binds to Omi/HtrA2 neutralizing its inhibitory function towards XIAP. Consequently, XIAP is free to bind to active caspase-3 and active caspase-9 thus preventing apoptosis. Induction of other apoptotic markers as caspase-8 activation, PS exposure and formation of microparticles do not or not exclusively involve the intrinsic pathway via Omi/HtrA2. Also, we observed a stronger decrease of activated caspase-3 than of activated caspase-9 since caspase-3 is acting at the very end of the apoptotic signaling cascade amplifying apoptotic events. Taken together, these findings demonstrate that Ucf-101 inhibits apoptosis in platelets and prove that platelets have a functional intrinsic apoptotic signaling pathway.

With the present study we demonstrate that platelets contain and release Omi/HtrA2 and Smac/Diablo from the IMS upon apoptotic stimuli. Importantly we were able to show that in platelets, as previously well studied in nucleated cells, Omi/HtrA2 inhibits the caspase

antagonist XIAP. This new insight in platelet physiology might be of importance for the understanding of the pathophysiology of ITP, as we have shown previously activated caspases in this disease [28] but also for the development of new anti-cancer agents that target anti-apoptotic proteins such as Smac mimetics or BH3 mimetics as ABT-737 and ABT-263 [23, 42].

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Authorship and Disclosures

JW and OS: designed the study; JW: performed all experiments; JW, OS: analyzed data; MLR provided essential advice and expertise with platelet handling; OS, JW, MLR and MS: wrote the manuscript. The authors have no conflicts of interest to declare.

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SUPPLEMENT

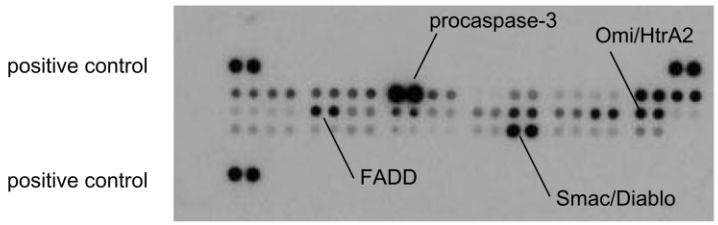
Apoptotic proteins in platelets and their interaction

In this study we demonstrated that platelets contain Omi/HtrA2 and XIAP, two proteins of the intrinsic apoptotic signaling pathway. To identify also other proteins involved in the intrinsic as well as extrinsic apoptotic-signaling pathway in platelets, we systemically investigated pro- and anti-apoptotic by Western blot and immunoprecipitation. To analyze apoptotic proteins in human platelets, we first determined the relative levels of apoptosis-related proteins in platelets on a Human Apoptosis Array (Figure 1A). Analysis of dot blots showed strong presence of procaspase-3, clusterin, cytochrome c and Smac/Diablo in human platelets, as well as an average presence of Bad, Bax, Bcl-2, Bcl-X, HiF1α, Hsp27, Hsp60, Hsp70, Omi/HtrA2 and an indication for cIAP1, Fas and XIAP (Figure 1B). We did not detect a positive signal for TRAIL, TNFR1, livin nor survivin. Based on these results we analyzed the expression of the previously mentioned apoptotic proteins as well as additional ones in human PRP (Figure 2) as well as in washed platelets (Figure 3) by Western blot. We found that human platelets express the death domain FADD and the BH3 interacting domain Bid (Figure 3A). FADD is a mediator of the extrinsic apoptotic-signaling pathway that transmits apoptotic signals from death receptors such as TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor) or CD95 (Fas receptor) to procaspase-8 recruiting caspase-8 [1]. Bid is processed to a truncated form by active caspase-8 and localizes to the mitochondria thus connecting the extrinsic to the intrinsic pathway. Death receptors as well as their death ligands were not detected on the protein level apart from the decoy receptor DcR2 [2] and FADD has not been analyzed. We were unable to detect the death receptors DR3, DR5 and TNFR1 as well as the Fas ligand in human platelets. We confirmed also the presence of Bcl-X, Bax and Bad that are well characterized in platelets and identified the inhibitor of apoptosis, cIAP-1 (Figure 2A & C). Furthermore, we found that platelets contain the heat shock proteins Hsp27, Hsp60 and Hsp70 (Figure 2B) as well as PON2 and clusterin (Figure 2C). PON2 is an anti oxidative protein that in nucleated cells was shown to have a protective thus anti-apoptotic effect in endoplasmic reticulum (ER) stress-induced apoptosis but only when the Ca^{2+} homeostasis is maintained [3]. The anti-apoptotic effect of the heat shock proteins Hsp27, Hsp60 and Hsp70 were studied in human cell lines. Hsp27 was shown to inhibit cytochrome c mediated activation of caspases by binding to released cytochrome c which prevents formation of the apoptosome [4]. In another study, Hsp27 decreased apoptosis by inhibiting Bax oligomerization and translocation [5]. Also Hsp60 was considered to have a similar anti-apoptotic role by preventing Bax from oligomerization [6]. Another study demonstrated that mitochondrial procaspase-3 is present in a complex of the chaperones Hsp60 and Hsp10 while induction of apoptosis dissociated procaspase-3 from both heat shock proteins [7]. Hsp70 on the other hand was shown to interact with the recruitment

domain (CARD) of Apaf-1 [8] [9]. This interaction inhibits the apoptosome formation and activation of procaspase-9 [8] [9]. Another protein considered to be involved in apoptosis is clusterin [10]. Clusterin exists as a secreted form that may be pro-apoptotic [10] and as a nuclear form that has an anti-apoptotic role by interfering with Bax [11]. Additionally, to depict all pro- and anti-apoptotic proteins that we have so far found in platelets, included in Figure 2A & 3A are those apoptotic proteins that we already mentioned previously (Chapter 4, Figure 2): Omi/HtrA2, Smac/Diablo, cytochrome c, Apaf-1, caspase-3, caspase-7 and XIAP.

Analysis of a Human Apoptosis Array containing control PRP and aged PRP indicated that aged platelets have a higher expression of Bad, cleaved caspase-3 as well as less FADD, Bcl-X, Hsp27 and cIAP (Figure 1B). In contrast to resting platelets, aged platelets expressed the death receptor TRAIL R1/DR4 (Figure 1B).

A)



B)

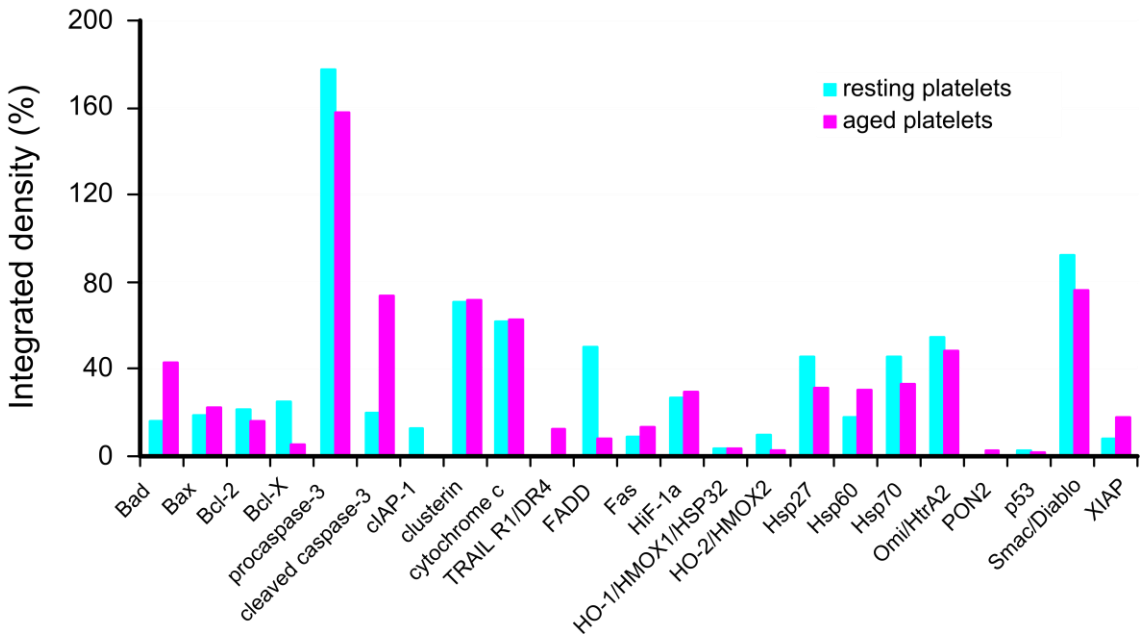


Figure 1. Platelets contain apoptotic proteins of the intrinsic and extrinsic signaling pathway. Platelets isolated from fresh **(A)** and aged PRP were analyzed on a Human Apoptosis Antibody Array for the presence of apoptotic proteins. Aged platelets were from the same donor as resting platelets and were stored for 7 days in capped tubes at RT. The integrated densities of the dot blot assays were calculated according to Dot blot analysis (ImageJ) and were adjusted to the positive control (representing 100%) **(B)**. Representative data of two different donors.

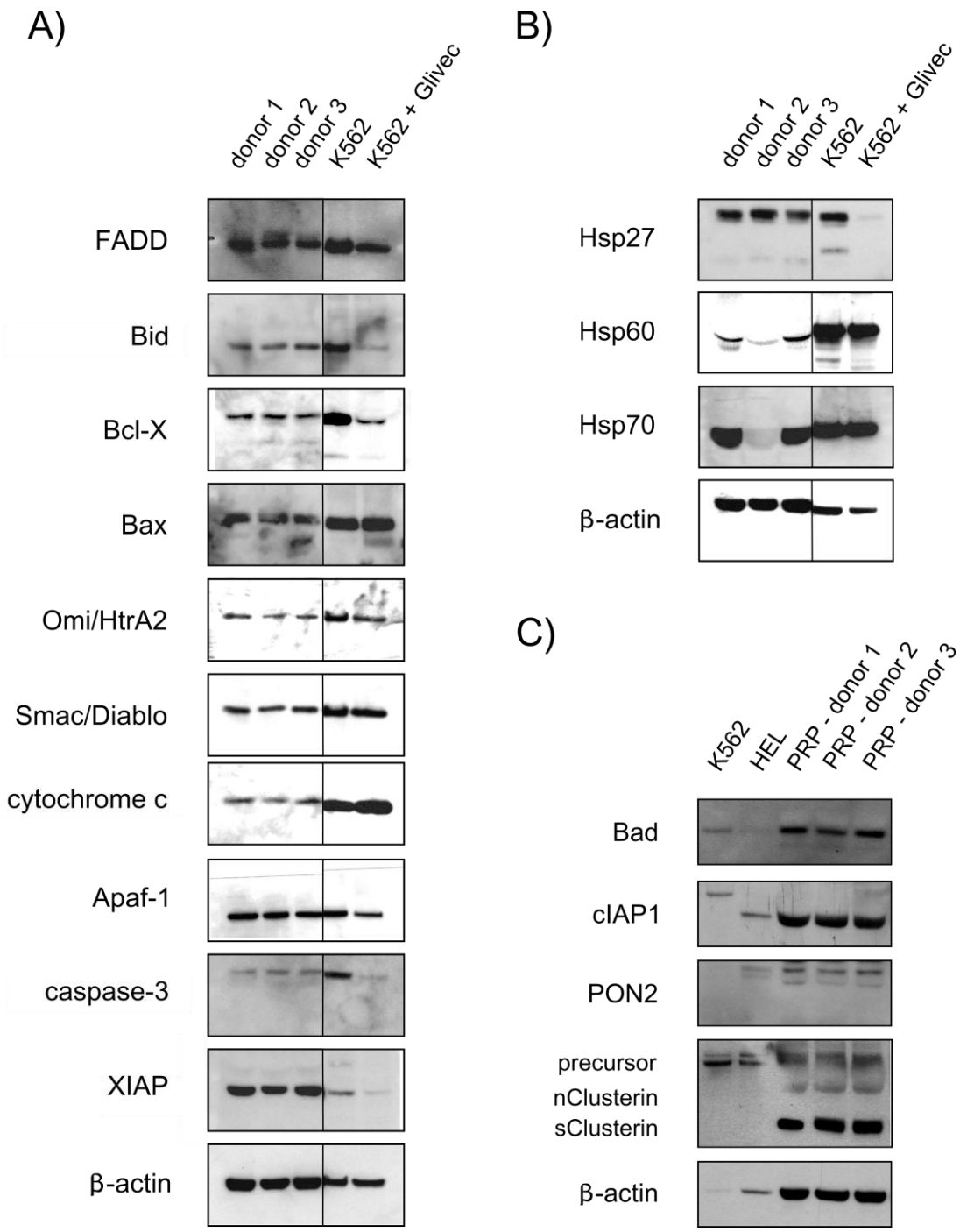


Figure 2. Platelets express the necessary components for a functional intrinsic apoptosis pathway. Western blot analysis of PRP using antibodies as indicated and described in materials and methods. Lysates from K562 or HEL cells were used as a positive control and lysates from K562 cells treated with Glivec as a control for apoptosis. Apoptotic platelet proteins of the extrinsic and intrinsic signaling pathway **(A)**. Heat shock proteins in platelets **(B)**. Further apoptosis-related proteins **(C)**. A representative β-actin was included as loading control.

To elucidate whether in activated platelets an increased or decreased expression of apoptotic proteins is observed we investigated their expression in platelets stimulated with C+T as well as with A23187 by Western blot (Figure 3). To have a control for apoptosis, K562 cells were treated with the cancer drug Glivec that induces cell death. Glivec-treated K562 cells presented less protein expression of Bid, Bcl-X, procaspase-3, XIAP and Hsp27. In most stimulated platelets we were not able to confirm any differences in the expression of apoptotic proteins apart from Bcl-X, caspase-9, caspase-7 and Hsp27. The cleaved fragment of caspase-9 was detected in platelets stimulated with C+T as well as with A23187 and the level of procaspase-7 decreased after stimulation of platelets with A23187. Bcl-XL was not present in platelets stimulated with A23187 consistent with the fact that Bcl-XL is degraded during platelet apoptosis [12]. For washed platelets, we detected two Bcl-X bands; the 30kDa band corresponding to Bcl-XL and a smaller band probably corresponding to the 15kDa pro-apoptotic Bcl-XS. Also in PRP, we were able to detect the smaller fragment of Bcl-X. In A23187-stimulated platelets, not only the anti-apoptotic Bcl-XL was decreased but also the pro-apoptotic fragment Bcl-XS. In A23187-stimulated platelets, we observed a decreased protein expression of FADD (3 independent experiments), as well as of Bax (2 independent experiments) (Figure 3A). Also Glivec-treated K562 cells had less FADD protein (Figure 3A). Interestingly, ABT-737-stimulated platelets showed also less FADD but more than A23187-stimulated platelets (Figure 3C). In nucleated cells, it has not been reported that neither FADD nor Bax are degraded during apoptosis. In activated platelets Hsp27 is no longer present similarly to apoptosis-induced K562 cells (Figure 3B). However, in washed platelets we detected a smaller fragment (15kDa) of Hsp27 than the expected 27kDa. A23187-stimulated platelets presented decreased expression of Hsp27 (4 independent experiments) compared to ABT-737 (two independent experiments). To compare platelet apoptosis with platelet aging, Western blot analysis was also performed of aged platelets. We detected less Hsp27 (27kDa) and less FADD (Figure 3C) as well as less Bcl-X and the active form of caspase-3 (data not shown) in aged platelets. However, aged platelets analyzed by Human Apoptosis Array presented less FADD than freshly isolated platelets (Figure 1B).

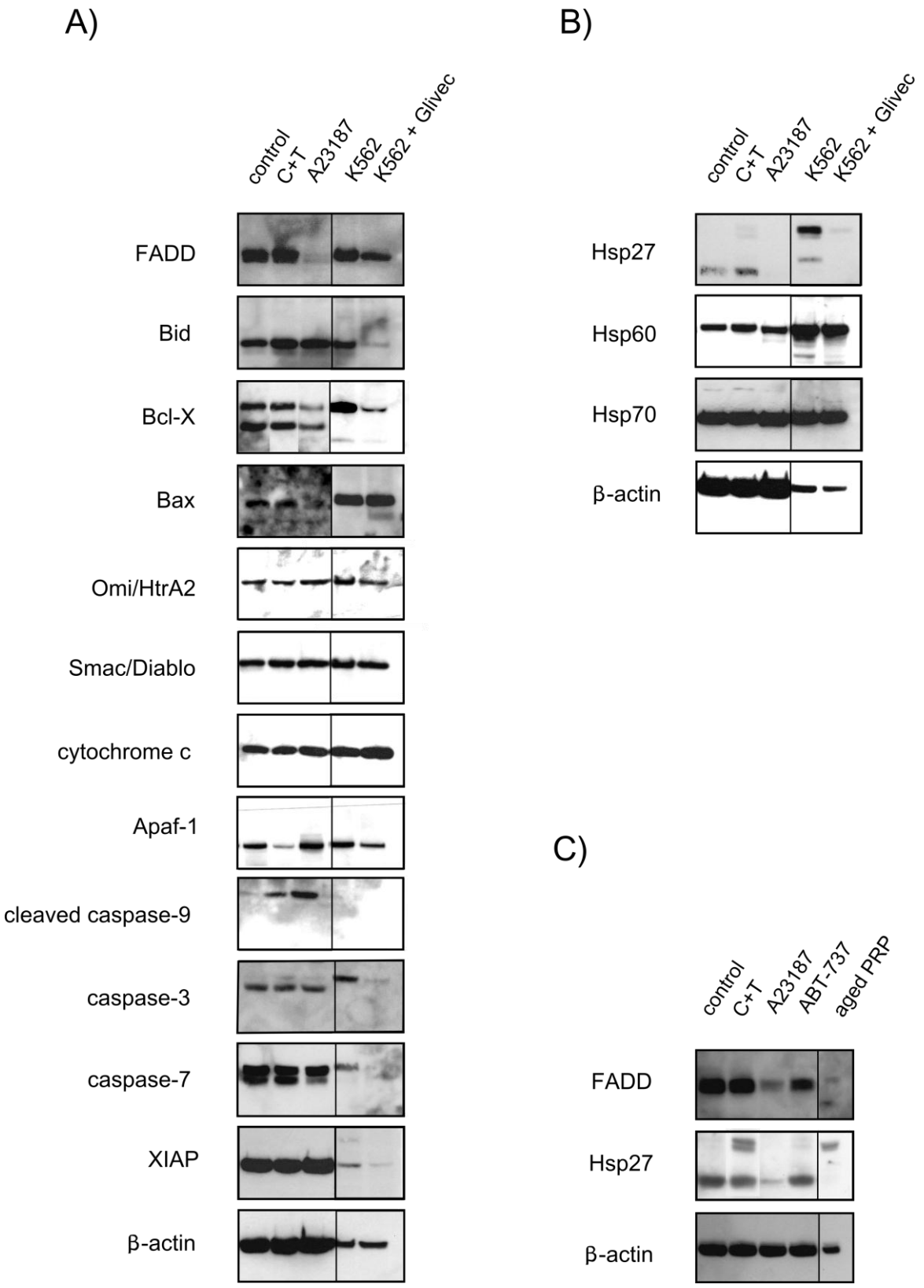

A)
control
C+T
A23187
K562
K562 + Glivec
FADD
Bid
Bcl-X
Bax
Omi/HtrA2
Smac/Diablo
cytochrome c
Apaf-1
cleaved caspase-9
caspase-3
caspase-7
XIAP
β-actin
B)
control
C+T
A23187
K562
K562 + Glivec
Hsp27
Hsp60
Hsp70
β-actin
C)
control
C+T
A23187
ABT-737
aged PRP
FADD
Hsp27
β-actin

Figure 3. Activated platelets only show minimal changes in the expression of apoptotic proteins. Western blot analysis of washed platelets using antibodies as indicated and described in materials and methods. Washed platelets (10^9/mL in Tyrodes-Hepes buffer) were either stimulated with collagen (10 μg/mL) and thrombin (1 U/mL) for 5 min, Ca^{2+} ionophore A23187 for 10 min (3 μM), or ABT-737 (3 μM) for 2 hr. For aging platelets, PRP was left in capped tubes for 7 days at RT. Lysates from K562 or HELA cells were used as a positive control and lysates from K562 cells treated with Glivec as a control for apoptosis. Expression of apoptotic proteins in platelets stimulated with C+T as well as A23187 or ABT-737 **(A-C)**. A representative β-actin was included as loading control.

To get a better understanding of the apoptotic signaling pathway in platelets, as well as the interaction of apoptotic proteins, we performed immunoprecipitations of several apoptotic proteins in human platelets. Apaf-1 co-immunoprecipitated with cytochrome c as well as with the anti-apoptotic protein Bcl-X (Figure 4A). Moreover, Bcl-X interacted with the two pro-apoptotic proteins Bax and Bad (Figure 4B). We also precipitated caspase-3 but were not able to detect any binding partners such as XIAP or cIAP-1 (Figure 4C). To compare the interactions of apoptotic proteins in resting platelets with those of activated platelets, we performed immunoprecipitations of platelets stimulated with C+T as well as with A23187. IPs of Bcl-X showed less Bcl-X protein in C+T-stimulated platelets and no Bcl-X protein in A23187-stimulated platelets consistent the protein amounts of the inputs and with the fact that Bcl-X is degraded during apoptosis [12] (Figure 4D). In nucleated cells, immunoprecipitation verified an association of Bad with Bcl-XL [13]. Furthermore, Apaf-1 co-immunoprecipitated with Bcl-XL and caspase-9 [14] and XIAP associated with the cleaved fragment of caspase-3 as well as caspase-7 [15]. The inhibitory effect of Omi/HtrA2 towards XIAP was shown by a direct association of Omi/HtrA2 with XIAP [16]. Moreover, an interaction of Apaf-1 with Hsp70 was demonstrated by IP [9].

To conclude, platelets express all necessary apoptotic proteins of the intrinsic apoptotic pathway to have a functional apoptotic-signaling. Besides, there is indication of an extrinsic apoptotic signaling pathway shown by the presence of FADD. Co-immunoprecipitation of Bcl-X with Bax and Bad in resting platelets verifies that Bcl-X inhibits the pro-apoptotic factors Bax and Bad when there is no onset of apoptosis. Furthermore, platelets contain the heat shock proteins Hsp27, Hsp60 and Hsp70 that in nucleated cells have been shown to interact with apoptotic proteins.

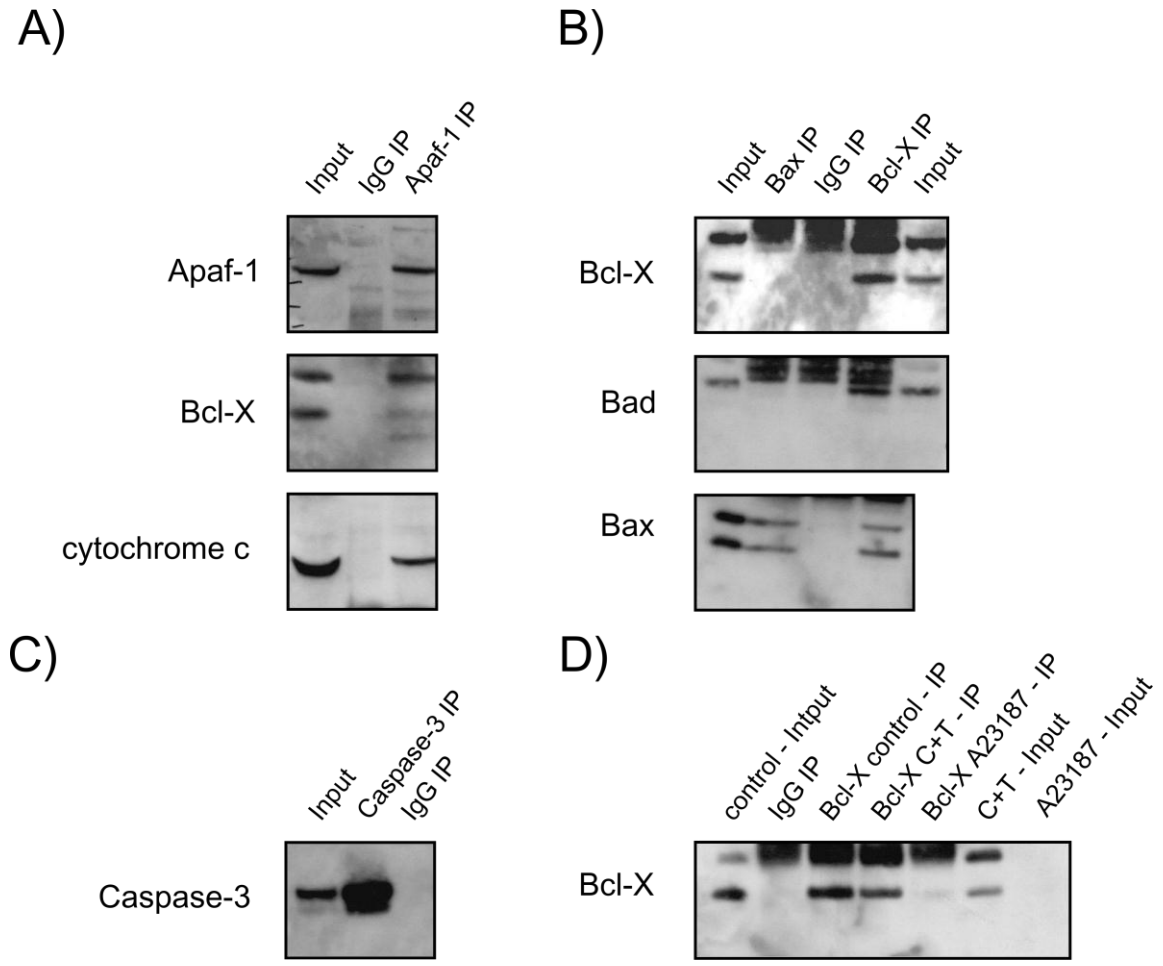


Figure 4. In platelets, Apaf-1 interacts with cytochrome c and caspase-9; Bcl-X complexes with Bad and Bax. Immunoprecipitations of apoptotic proteins in platelet concentrates were performed according to the IP protocol followed by their detection by Western blot. IP of Apaf-1 probed for associated proteins **(A)**. IP of Bcl-X probed for associated proteins **(B)**. IP of caspase-3 **(C)**. IP of Bcl-X in platelets stimulated with C+T as well as with A23187 compared to the corresponding inputs **(D)**. As a control, the relevant non-specific mouse, goat or rabbit immunogobulins G or A (IgG, IgA) were used.

Materials and methods

Materials

ABT-737 was from Active Biochemicals (Maplewood, USA). Glivec was provided by Dr Jeroen Goede, University hospital Zürich.

For Western blot, in addition to the antibodies described in the beginning of Chapter 4, anti-human Bax rabbit pAb from Cell Signaling Technology (Boston, MA, USA); anti-human Bcl-X rabbit pAb, anti-human Bid rabbit pAb, anti-human Bad rabbit pAb, anti-human FADD goat pAb, anti human Hsp27 rabbit pAb, anti-human Hsp60 mouse pAb, anti-human Hsp70 mouse mAb (clone 242707), anti-human cIAP1 goat pAb, anti-human PON2 goat pAb from R&D Systems (Minneapolis, MN USA) were used. For immunoprecipitations, anti-human caspase-3 goat pAb (clone CPP32), anti-human Bcl-X rabbit pAb and anti-human Apaf-1 mouse mAb (clone 94408) from R&D Systems (Minneapolis, MN USA) were used.

Human Apoptosis Array

To simultaneously detect the relative expression of 35 apoptosis-related proteins in a single sample, a Human Apoptosis Antibody Proteome Profiler™ Array (R&D Systems, Minneapolis, MN USA) was used according to its manual. The array contained capture antibodies spotted in duplicates on a nitrocellulose membrane. As a lysed cellular extract we used platelets from freshly isolated PRP and from 7 days aged PRP (stored at RT) of the same donor. Diluted cellular extracts were incubated over night on the Human Apoptosis Array. The array was washed to remove unbound proteins, followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied and a signal was produced at each capture spot corresponding to the amount of protein bound. Dot blots were scanned with a brother MFC-8880DN printer using a resolution of 600 dpi. To analyze, we determined the pixel density of each spot (ImageJ, Dot blot analysis).

Immunoprecipitation (IP)

Preparation of platelet lysates: 15 mL PRP from platelet concentrates were supplemented with 5 mM EDTA and centrifuged for 20 min at 500g without breaking. The pellet was snap frozen in liquid nitrogen. For lysis, the pellet was thawed on ice and 3 volumes of polysome lysis buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Igepal, 20 mg/mL Heparin, protease inhibitors) were added. The extract was pipetted several times and centrifuged at 14'000g for 10 min at 4°C. The supernatant was removed, transferred to a new tube and centrifuged again twice to remove the lipid layer on top of the supernatant. Protein concentration was determined by Bradford and 20 mg protein cell extract aliquots were prepared.

Preparation of antibody coating bead mix: For each IP 50 µl of drained protein G (for mAbs) or A (for pAbs) Sepharose beads (GE Healthcare, Glattbrugg, Switzerland) were used. The ethanol was removed by centrifuging at 10'000 rpm for 3 min at 4°C. Beads were washed once in 8 volumes of NT2 buffer (50 mM Tris HCl pH 7.5, 300 mM NaCl, 5mM $MgCl_2$, 0.05% Igepal, protease inhibitors) and twice with NT2-Ab buffer (NT2 buffer supplemented with 50mg/ml BSA, 20 mg/mL Heparin). Beads were resuspended in 8 volumes of NT2-Ab. 20 µg of the immunoprecipitating antibody was added and incubated over night on a rotating device at 4°C.

Immunoprecipitation: The beads were washed 4 times with 1 mL NT2 buffer per assay by centrifuging at 10000 rpm for 3 min at 4°C and kept on ice. The lysate was thawed on ice for 10 min and centrifuged at 14'000g for 10 min at 4°C. The antibody-coated beads were resuspended in 10 mL NT2-RIP buffer (NT2 buffer supplemented with 2mM DTT, 1 g/L sodium azide and 20 mg/mL Heparin) - the volume of resuspended beads in NT2 buffer corresponds to 10 times the volume of the lysate – and the pellet lysate was added. The IP reaction was tumbled on a rotation device for 6 hr at 4°C. After incubation beads were centrifuged at 2000 rpm for 2 min at 4°C. 1 mL ice-cold NT2 buffer was added and the washing step was repeated 3 times. The washed beads were resuspended in 500 µl SDS-EDTA solution (50 mM Tris HCl pH 8, 10 mM EDTA, 1% SDS, 100 mM NaCl) and incubated at 65°C, 900 rpm, for 10 min. The beads were centrifuged at 10'000 rpm for 3 min at RT and the supernatant was saved for Western blot analysis.

Authorship

JW and OS: designed the study; JW: performed all experiments of the supplement

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CHAPTER 5

Expression patterns of Th1 and Th2 cytokines in pediatric immune thrombocytopenia at presentation and their modulation by IVIg

Jeannine Winkler*[1,2], Sabine Kroiss[1], Jana Pachlopnik*[3], Markus Schmugge[1], Oliver Speer*[1,2]

* authors contributed equally to the work

[1]*Division of Hematology and Children's Research Center, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland*

[2]*Zurich Center for Integrated Human Physiology, University of Zurich, Switzerland, University of Zurich, Switzerland*

[3]*Division of Immunology and Children's Research Center, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland*

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Correspondence: Dr. Oliver Speer, Division of Hematology, University Children's Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland
E.Mail: oliver.speer@kispi.uzh.ch

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Summary

To evaluate the role of cytokines in pediatric immune thrombocytopenia, we investigated plasma of 10 pediatric patients with acute immune thrombocytopenia (ITP), before and after IVIg treatment. Healthy children with platelet counts in the normal range were enrolled as controls and children with a non-immune thrombocytopenia as a control for the low platelet count. All ITP patients presented with platelet counts < 25 x 10^9/L and bleeding symptoms. Plasma concentrations of 42 cytokines/chemokines were analyzed by Luminex technology measurements of all four groups. ITP patients had significantly increased levels of the Th1 cell commitment cytokines TNF-α, IFN-γ and IL-2 as well as of the Th2 cytokines IL-6, IL-10 and IL-13 that are involved in the B cell differentiation. After IVIg treatment, platelet counts increased to > 20 x 10^9/L in all patients; the observed elevated plasma cytokine levels decreased after therapy but the differences were not statistically significant. In conclusion, in acute pediatric ITP, increased plasma cytokine levels are observed that are modulated by IVIg.

Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune disease, mainly caused by autoantibodies against platelet surface receptors leading to an enhanced clearance of platelets. ITP patients are treated with intravenous immunoglobulins (IVIg) or other medications such as corticosteroids, anti-D antibodies or CD20 antibodies that ameliorate thrombocytopenia after 24-48 h of administration. Apart from auto reactive B cells [1] also CD4+ T-helper (Th) cells as well as their cytokines have been associated with ITP, especially with chronic ITP [2] [3]. Th cells are essential players in immune responses and inflammatory diseases that after stimulation by antigen presenting cells differentiate into distinct lineages as Th1, Th2 or Th17 cells. Cytokines and chemokines function as cell signaling molecules in inflammation, immunity as well as in the hemostatic balance and are produced by different cell types such as T cells, macrophages or monocytes. In ITP, increased plasma levels of IL-2, IFN-γ and/or IL-10 were reported to be associated with chronic ITP [4] [5]. In contrast, Semple et al. did not detect increased plasma concentrations of IL-2, IFN-y and IL-10 in acute ITP patients [4] and other groups mainly analyzed cytokines in chronic ITP patients. A recent study showed that chronic ITP patients have reduced serum expression levels of the transforming growth factor β-1 (TGF-β1) [6]. Additionally, higher IL-17 and IFN-γ plasma levels were found and a positive correlation of IL-17 with IFN-γ [6]. Th17 cells have been found to be more potent than Th1 cells in inducing autoimmune diseases [7]. TGF-β1 induces IL-17 helper cells, and is considered to be an inhibitor of B cell proliferation and autoantibody production as well as of megakaryocytes maturation [8]. It was suggested that increased plasma concentrations of IL-17 and IFN-γ may be responsible for a dysregulation of cellular immunity in chronic ITP [6]. Furthermore, the negative correlation of TGF-β1 with platelet counts indicated that TGF-β1 might be one of the factors observed in chronic ITP [6]. Also in another study increased plasma cytokine levels of IL-17 were determined in chronic ITP [9]. Besides, increased levels of IL-1β, IL-6 and IL-23, cytokines involved in Th17 cell activation and maintenance, were detected [9]. Furthermore, chronic ITP patients presented with elevated plasma cytokine levels of IFN-γ, IL-2, TNF-α, IL-12p-70 and IL-10 but no with TGF-β nor IL-4 [9]. Additionally, acute ITP patients as well as thrombocytopenic patients in general presented increased granulocyte-macrophage colony-stimulating factor (GM-CSF) plasma levels but not elevated IL-3 and IL-6 [10]. GM-CSF, as IL-3, is involved in the formation of megakaryocyte colonies while IL-6 is a late factor in megakaryocytes differentiation [11]. The B cell activating factor (BAFF) belongs to the TNF ligand family and is crucial for the maintenance of B cell development [12]. In a study of chronic ITP, higher levels of BAFF were measured in patients suffering from a chronic active ITP [13] [14] .

However, in ITP, the data on polarization of the immune system towards Th1, Th2 or Th17 cells are contradictory and results are not consistent. In an earlier study of chronic ITP patients, no difference in the levels of IL-17, TGF-β, IL-6 nor IFN-γ were found compared to controls [15] while others reported only an involvement of the Th1 cell commitment and not of the Th2 commitment [5]. To extend these previous observations in acute ITP patients, we characterized the cytokine panel in ITP patients and studied whether increased cytokine levels occur in plasma of pediatric patients with primary ITP, whether their plasma levels change by treatment with IVIg. In this study we demonstrated that acute ITP patients at diagnosis have increased plasma levels of Th1 cytokines IFN-γ, TNF-α and IL-2 as well as of the Th2 cytokines IL-6, IL-10 and IL-13. In conclusion, we confirmed that in ITP patients macrophages as well as B and T cells are activated.

Materials and methods

Patients

In this prospective study, approved by the local ethics committee, 10 children (4 females and 6 males) with newly diagnosed ITP were investigated after obtaining written informed parental consent (Table 1 and 2). Patients were recruited in the Children's Hospital Zurich within four years and followed during one year. All patients fulfilled the criteria for primary ITP [16]. The median age at diagnosis was 3.4 years (range: 1.6 – 6.5 years). 3 patients had signs of a respiratory tract infection without fever at the date of diagnosis, one of them presented with urtikaria. One further patient was still under antibiotic treatment because of streptococcal angina. In 2 patients, ITP was preceded by 2–4 weeks by a viral gastroenteritis. In 4 patients, there was no history of infection. All patients had a very low platelet count median 3 x 10^9/L (range <1 to 22 x 10^9 /L) at diagnosis. The severity of bleeding symptoms at diagnosis was graded with a standardized bleeding score according to Buchanan and Adix [17] and Bolton-Maggs and Moon [18], which was modestly adapted. Bleeding scores were 2 to 3 with most patients showing large hematoma and multiple petechiae. Three patients presented with mucosal bleeding, one of them with severe epistaxis and hematemesis, in a second patient a tongue lesion was bleeding persistently and a third patient had bloody stools. 10 patients were treated with IVIg at a dose of 0.8 g/kg body weight and one patient received a second dose of IVIg.

9 healthy control children, 4 females and 5 males, median age of 15.9 years (range: 0.8 – 18.1 years), with no history of autoimmune disease, coagulopathy, transfusion of blood products, or of ongoing medication, were recruited from the outpatient clinic after obtaining written informed parental consent. To control effects due to the thrombocytopenia itself, in addition, 9 children, 7 females and 2 males, median age 10.3 years (range: 4.2 – 15.3 years), 1 patient with a thrombocytopenia due to aplastic anemia and 8 with a chemotherapy-related thrombocytopenia (cTP), were recruited after obtaining written informed parental consent. Their median platelet count was 12 x 10^9/L (range: 3 – 53 x 10^9L).

Table 1.

Clinical and laboratory characteristics of children with primary ITP: bleeding scores and platelet count results before and after IVIg treatment are presented for each individual patient as well as platelet count at diagnosis and after treatment, infection prior or at time of diagnosis as well as patient's history. Patients who recovered from ITP are defined as in remission; patients who had a low platelet count after 3 months were defined as persistent ITP and who suffered from ITP for longer than 6-12 months as chronic ITP.

Patient #	Age (years)	Sex	Bleeding score	Platelet count at diagnosis ($x10^9$/L)	# of IVIg doses (0.8 g/kg)	Platelet count last IVIg dose ($x10^9$/L)	Infection prior or at time of diagnosis	History
1	6.5	F	2.5	3	1	54	streptococcal angina, penicillin treatment	chronic
2	3.5	F	2	2	2	45	respiratory tract infection, w/o fever, with urtikaria	chronic
3	2.5	M	2.5	6	1	24	respiratory tract infection, w/o fever	remission
4	3.8	M	2.5	8	1	24	no infection	remission
5	5.8	M	2.5	1	1	47	gastroenteritis (2-3 weeks before diagnosis)	remission
6	2.1	M	2	<1	1	31	no infection	remission
7	4.2	F	3	1	1	22	no infection	remission
8	1.6	F	2	2	1	38	no infection	persistent
9	3.2	M	2	22	1	46	gastroenteritis (4 weeks before diagnosis)	remission
10	2.8	M	3	14	1	28	respiratory tract infection, w/o fever	remission
median	3.4	-	2.5	3	1	35		

Table 2.

Plasma cytokine levels of IL-6, IL-10, IL13, TNF-α, IFN-γ, IL-1ra, GM-CSF and MIP-1α as well as proportions of platelets with activated caspase-3 (aCASP3), caspase-8 (aCASP8) and caspase-9 (aCASP9) in pediatric ITP patients at diagnosis.

Patient #	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-13 (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-1ra (pg/ml)	GM-CSF (pg/ml)	MIP-1α (pg/ml)	aCASP3 (%)	aCASP8 (%)	aCASP9 (%)
1	2	6	0	9	2	0	48	0	18.2	42.7	20.9
2	1	6	2	8	11	56	12	21	3.3	1.7	2.1
3	21	24	91	27	62	305	31	46	6.3	9.1	8.7
4	10	39	30	12	2	248	11	44	6.8	16.7	13.1
5	25	25	113	35	91	218	53	39	1.4	4.6	4.6
6	0	2	0	10	<1.1	0	2	0	7.6	20.7	13.1
7	13	7	57	26	57	178	42	29	6.3	15.5	21.4
8	26	13	102	49	107	161	50	35	5.1	3.5	2.2
9	0	5	0	9	3	0	1	6	nd	nd	nd
10	3	2	11	15	7	30	20	13	8.8	13.8	12.5
control median +/- SEM	0+/- 0.1	2.7+/- 0.7	0.5+/- 0.3	5.3+/- 0.6	5.9+/- 0.1	3.7+/- 2.2	7.6+/-0.9	5.4+/- 1.8	1.4+/- 0.5	0.9+/- 0.2	0.8+/- 0.2

Blood sampling and routine testing

Venous blood samples for flow cytometric analyses were taken from ITP patients at the time of diagnosis prior to treatment and 12 to 24 hr after the last treatment with IVIg (prior to discharge from the hospital). Blood samples were taken from healthy controls when inserting a venous line and from children with cTP prior to transfusion of a platelet concentrate. Samples were collected into citrate anticoagulant (final concentration, 10.5 mM). The total volumes of the blood samples taken did not exceed 1% of the total blood volume of the patients or the controls. Blood indices including platelet counts were measured using an automated blood counter, Sysmex XE-2100 (Sysmex Digitana, Horgen, Switzerland). Plasma samples were immediately frozen at -80°C.

Luminex analysis

Detection of 42 human cytokines/chemokines was done by Luminex® xMAP® Technology using MILLIPLEX® MAG multiplex immunoassays (Millipore, Rotkreuz, Switzerland). Preparation of plasma samples and measurements were performed according to the manual of Millipore. In short, plasma samples were assayed in duplicates and incubated in a 96-well solid plate with premixed magnetic beads, covalently coupled to a specific capture antibody, for 2 hr at RT. The plate was washed twice using a hand-held magnet. After incubation, biotinylated detection antibodies cocktail was added and reactions were incubated with shaking for 1 hr at RT. Subsequently, streptavidin-phycoerythrin solution was added and the reaction was incubated with shaking for 30 min at RT. The plate was washed twice and samples were resuspended in 150 µL sheath fluid. The fluorescent signal of the beads was read by a Bio-Plex 200 System with Bioplex Manager Software version 5.0. (Biorad, Reinach, Switzerland). The median fluorescent intensity data was analyzed using a 5-parameter logistic curve-fitting method and the cytokine/chemokine concentrations of the samples were calculated (in picogram per milliliter, pg/mL). Final analysis of the raw data was done by Milliplex Analyst Software (Millipore, Rotkreuz, Switzerland). The 42 cytokines/chemokines included in the multiplex immunoassay were: EGF, Eotaxin, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGF-α, TNF-α, TNF-β, VEGF, sCD40L, sIL-2Ra. Whisker blots of statistically significant cytokines of the Th1 cell commitment, Th2 cell commitment and of chemokines were generated.

Measurement of BAFF plasma levels

BAFF levels were measured using specific enzyme-linked immune assay (ELISA) according to the manufacturer's recommendations (Hoelzel Diagnostica, Köln, Deutschland).

Serum BAFF levels were calculated from a standard curve generated from recombinant human BAFF.

Statistical analyses

Statistical analysis between the 4 groups (ITP, ITP after IVIg, healthy controls, cTP) was done using Kruskal-Wallis one-way ANOVA, followed by Wilcoxon signed-rank test between pairs of medians. Correlation was assessed by the Spearman correlation coefficient. Medians were considered statistically significantly different for $p<0.05$. Analysis was done using SPSS (SPSS, Zurich, Switzerland).

Results

We recently found that ITP patients at diagnosis have increased proportions of platelets with activated caspase-3, -8 and -9 [19]. In this study we analyzed plasma cytokine levels in a subgroup of the previously included ITP patients.

To investigate the role of cytokines in children with acute ITP, we evaluated the plasma concentration of 42 cytokines in ITP patients at diagnosis and after IVIg compared to healthy controls and children with a non-immune thrombocytopenia (cTP). At diagnosis ITP patients had a platelet count of 3 x 10^9/L. All patients showed a rise in platelet count above 20 x 10^9/L in the next 24 to 48 hr after IVIg treatment, accompanied by declining bleeding symptoms (Table 1). From 10 patients blood was investigated at diagnosis of ITP and 24-48 hr after treatment with IVIg. After 6 months 5 patients were in remission with stable normalized platelet counts without further treatment while one patient achieved remission one year after diagnosis. 2 patients had persistent ITP defined as a low platelet count for longer than 3 months and were followed less than 1 year. 2 patients suffered from chronic ITP with two of them receiving treatment intermittently.

Due to the low platelet count (3 x 10^9/L) at diagnosis, ITP patients showed decreased levels of plasma cytokines that are produced by platelets such as RANTES and soluble CD40 ligand (sCD40L) (Figure 1A). After treatment of ITP patients, a significant increase in RANTES as well as sCD40L was detected. Furthermore, ITP patients at diagnosis presented decreased plasma levels of growth factors produced by platelets as the platelet derived growth factor PDGF-AA and PDGF-AB/BB as well as the growth regulated oncogene (GRO) (Figure 1B). After therapy, there was a significant increase in PDGF-AA and PDGF-AB/BB and a non significant increase in GRO. Also patients suffering from a thrombocytopenia due to chemotherapy had decreased plasma levels of RANTES, sCD40L, PDGF-AA and PDGF-AB/BB. Of this group, one patient suffered from aplastic anemia and therefore did not receive chemotherapy but presented thrombocytopenia; however, this patient had normal plasma levels of RANTES and sCD40L. As expected, RANTES, sCD40L, PDGFAB/BB and GRO correlated positively with the platelet count.

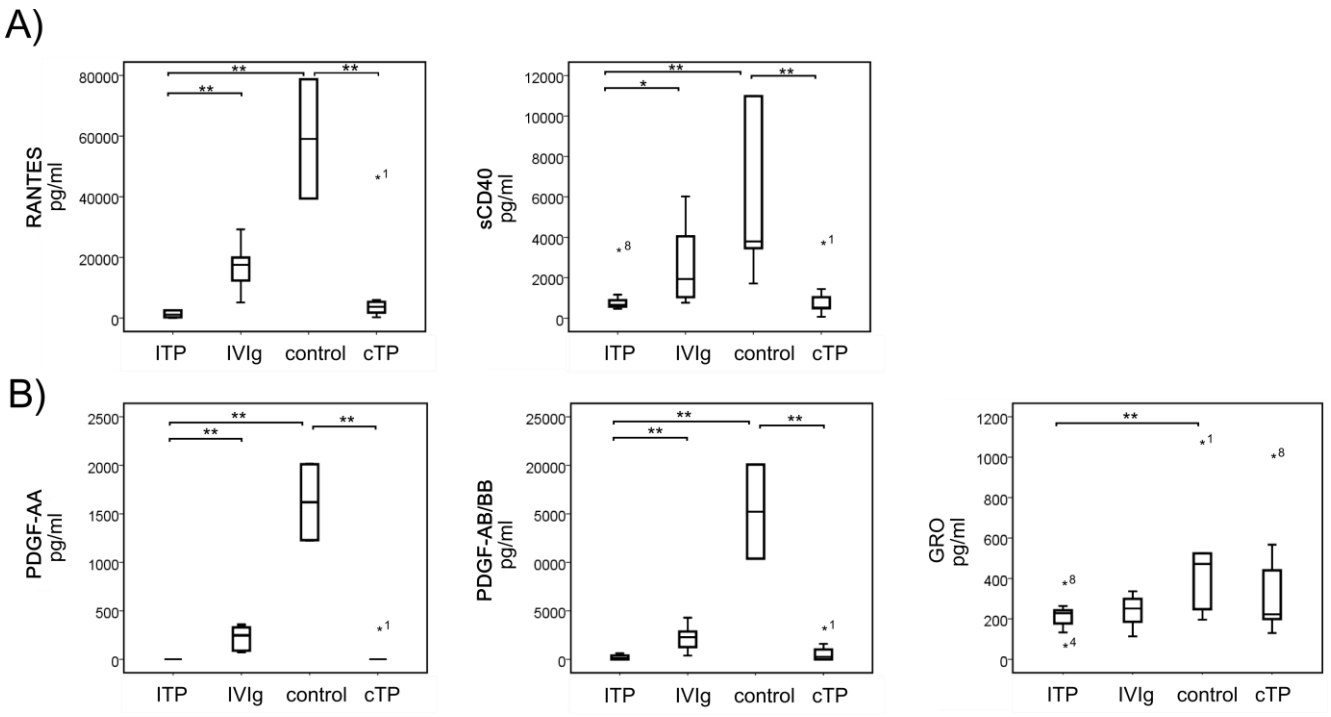

Figure 1. Platelet cytokines: ITP patients show clearly decreased levels of platelet-derived cytokines. Box plots representing the plasma levels of RANTES and SCD40L **(A)**, PDGF-AA, PDGF-AB/BB and GRO **(B)** in patients with acute ITP (n=10) at diagnosis and after therapy compared to controls (n=9) as well as cTP patients (n=9). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively; the horizontal bar across the box indicates the median and the ends of the vertical lines indicate the minimum and maximum data values * $p < 0.05$ and ** $p < 0.01$

ITP patients at diagnosis had significantly increased Th1 cytokine plasma levels as TNF-α, IFN-γ and IL-2 (Figure 2A) and elevated plasma levels of the Th2 cytokines IL-6, IL-10 and IL-13 (Figure 2B) compared to controls. Furthermore, ITP patients at diagnosis had significantly increased plasma levels of GM-CSF and of the IL-12 subunit IL-12p 40 compared to controls while the increase in the bioactive IL-12p 70 subunit was not significant (Figure 2C). 7 patients presented increased GM-CSF (> 12 pg/ml) plasma levels at diagnosis (Table 2) whereas after therapy only 3 patients had still elevated GM-CSF levels. Also cTP patients presented increased GM-CSF plasma levels, however, not statistically significant. IL-8 that is produced by macrophages was significantly increased in ITP patients. There was a tendency of an increase in the Th17 cytokine IL-17 as well as of IL-7, a cytokine involved in T cell expansion (Figure 2D). After treatment of ITP patients, the elevated plasma median concentrations of IL-2, IL6, IL-13, IFN-γ, IL-12 40P/70P, IL-8 and IL-17 decreased; however, medians were not statistically significant.

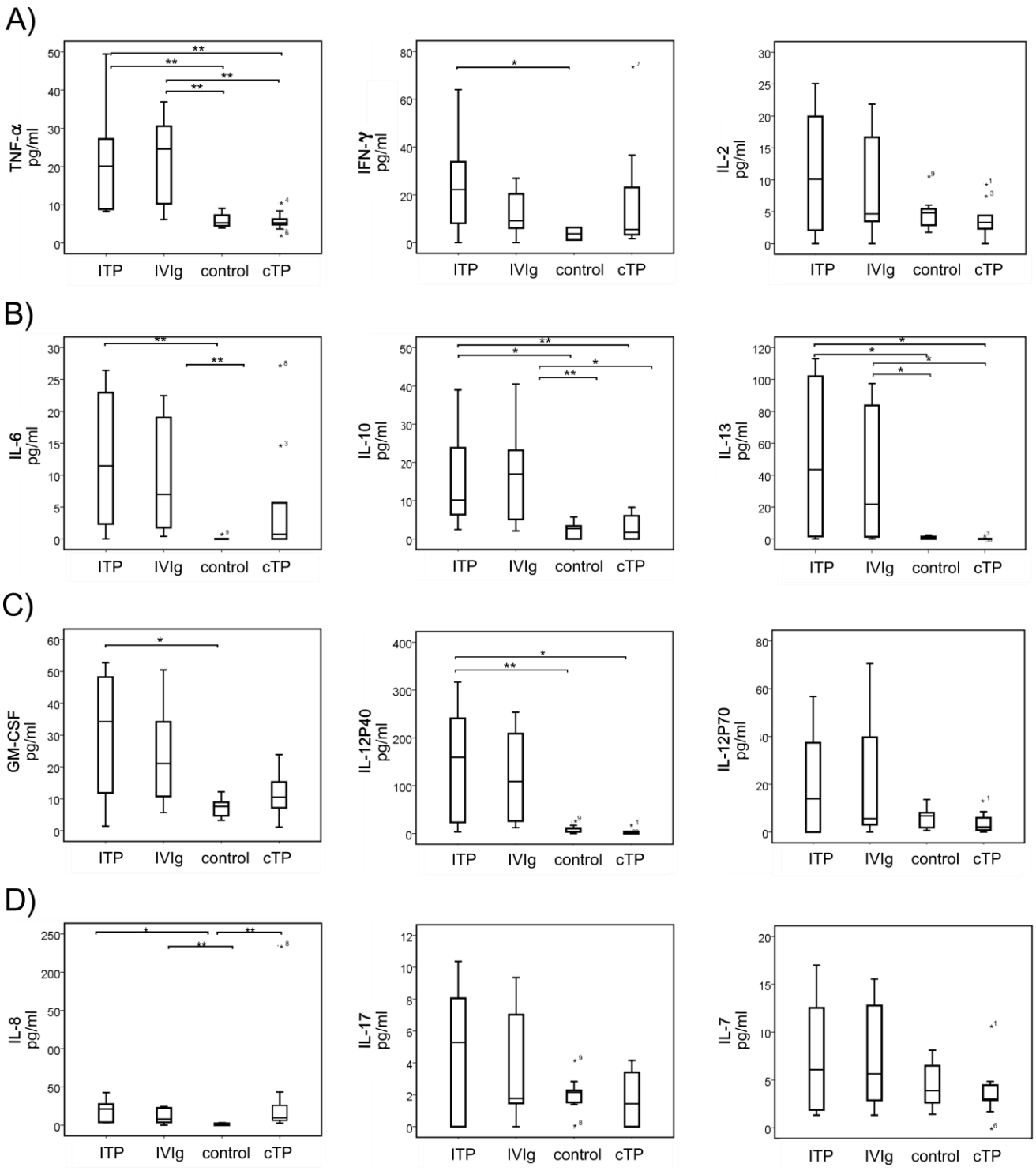
A)
TNF-α pg/ml
IFN-γ pg/ml
IL-2 pg/ml
B)
IL-6 pg/ml
IL-10 pg/ml
IL-13 pg/ml
C)
GM-CSF pg/ml
IL-12P40 pg/ml
IL-12P70 pg/ml
D)
IL-8 pg/ml
IL-17 pg/ml
IL-7 pg/ml
ITP
IVIg
control
cTP

Figure 2. Increased plasma levels of Th1, Th2 and Th17 cytokines in ITP patients: Box plots representing the plasma levels of Th1 cytokines **(A)** and Th2 cytokines **(B)**, cytokines produced by macrophages **(C)** as well as of IL-8, IL-17 and IL-7 **(D)** in patients with acute ITP (n=11) at diagnosis and after therapy compared to controls (n=9) as well as cTP patients (n=9). The upper and lower limits of the boxes represent the 75^{th} and 25^{th} percentiles, respectively; the horizontal bar across the box indicates the median and the ends of the vertical lines indicate the minimum and maximum data values * $p < 0.05$ and ** $p < 0.01$

Additionally, ITP patients at diagnosis also presented increased plasma levels of IL-1 as well as of its receptor antagonist IL-1ra and the soluble IL-2ra, the receptor agonist of IL-2 (Figure 3A). Besides, we detected increased plasma levels of chemokines: the interferon-γ induced protein 10 (IP-10) that is secreted in response to IFN-γ, the macrophage inflammatory proteins MIP-1α and MIP-1β, the macrophage-derived chemokine (MCD) and the monocyte chemoattractant protein-1 (MCP-1) that recruits monocytes to sites of infections (Figure 3B). All plasma levels of cytokines and chemokines described in Figure 3 did not present a significantly decreased median after treatment with IVIg apart from MIP-1β levels that were statistically increased after treatment.

We did not find a correlation between elevated levels of cytokines and chemokines and an ongoing or preceding infection at initial presentation of ITP nor revealed a connection to the patient's history (Table 1), suggesting that infections were not responsible for the increased plasma expression levels of the described cytokines/chemokines. In Table 2 we show the proportions of platelets with activated caspase-3, -8 and -9 together with the plasma cytokines levels of IL-6, IL-10, IL-13, IFN-γ, IL1ra, GM-CSF and MIP-1α. Patients with low plasma cytokine levels presented either high or low activated caspases.

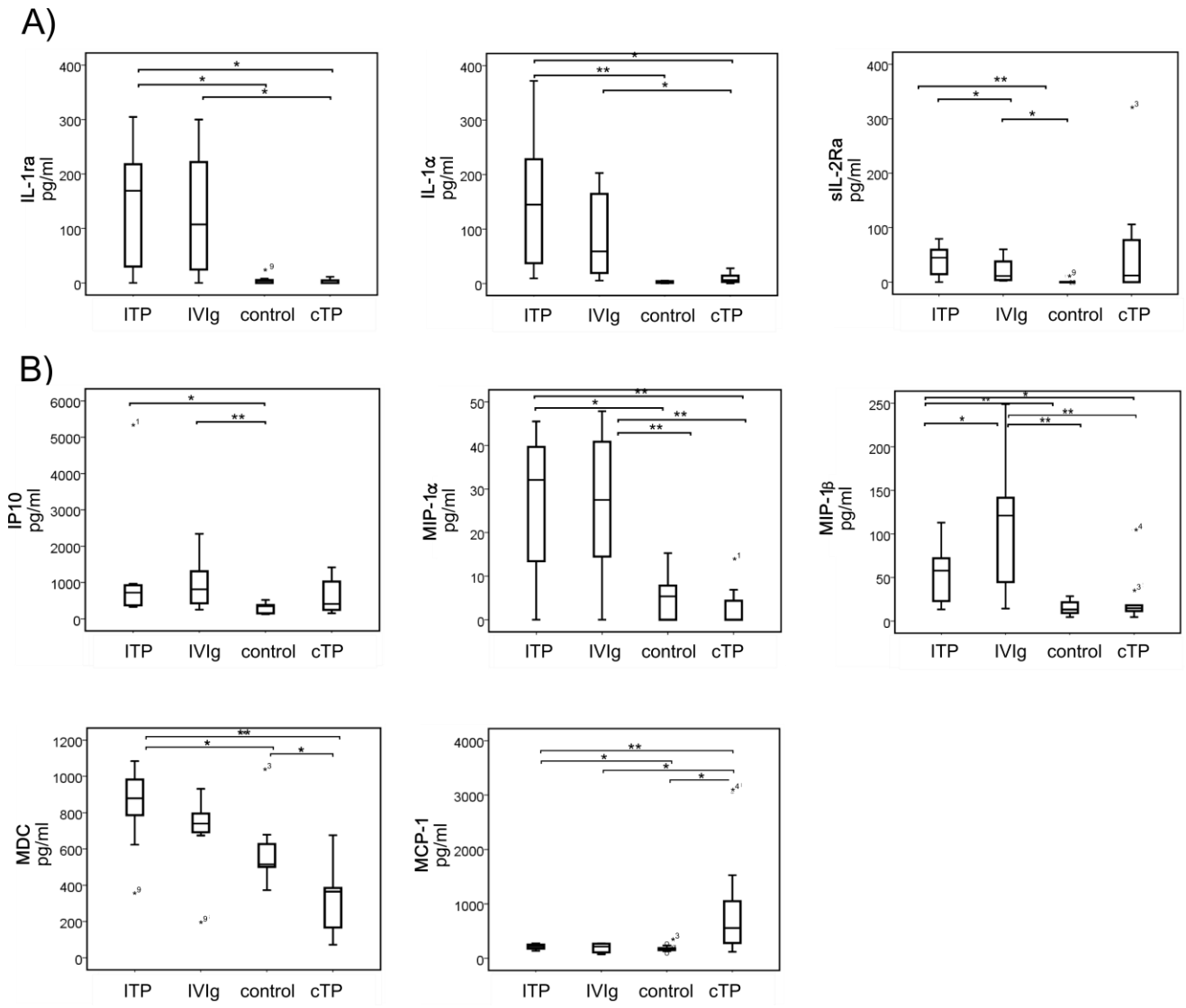

Figure 3. ITP patients have altered plasma levels of receptor agonist cytokines as well as of chemokines. Box plots representing the plasma levels of IL-1ra, IL1a, sIL-2Ra **(A)**. Box plots representing IP-10, MIP-1α, MIP-1β, MDC and MCP-1 **(B)** in patients with acute ITP (n=10) at diagnosis and after therapy compared to controls (n=9) as well as cTP patients (n=9). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively; the horizontal bar across the box indicates the median and the ends of the vertical lines indicate the minimum and maximum data values * $p < 0.05$ and ** $p < 0.01$

Apart from cytokines in Figure 1, cTP patients showed significantly increased plasma levels of the cytokines EGF, FGF-2, G-CSF, Flt-3 Ligand, IL-8, MCP-1 and VEGF compared to controls (data not shown) while MDC plasma level was statistically lower in cTP than in control (Figure 2 & 3). IL-8 and MCP-1 were increased in cTP as well as in ITP patients compared to healthy children. Cytokines statistically increased in ITP but not in cTP were TNF-α, IL-10, IL-13, IL-12p40, IL-1ra, IL-1α, MIP-1α and MIP-1β.

In our study population, acute ITP patients did not present increased levels of BAFF as described elsewhere [13] [20] [14] (Figure 4).

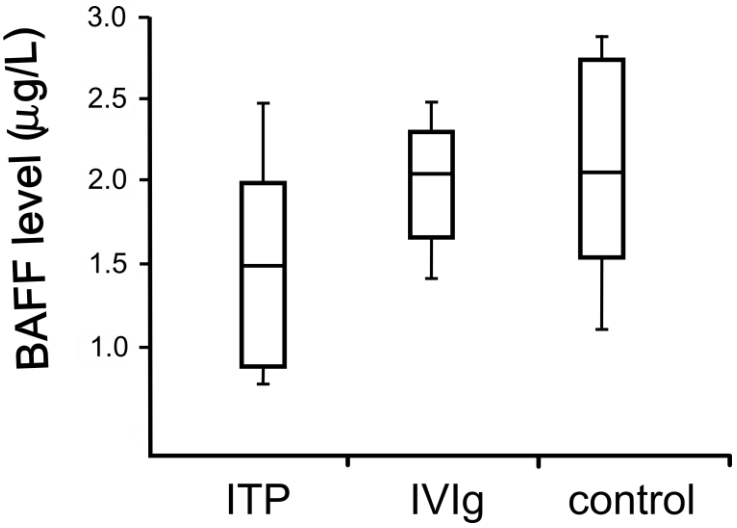

Figure 4. BAFF levels in ITP patients compared to control: ITP patients did not present statistically significant increased or decreased levels of B cell activating factor (BAFF) at diagnosis and after treatment compared to control. BAFF concentrations were measured in plasma by ELISA. n = 10 for ITP, IVIg as well as control

Discussion

In this study, we investigated cytokines/chemokines in a clinically well-defined group of children with ITP that have been investigated previously for platelet apoptotic manifestations [19]. All patients presented with bleeding symptoms including petechiae, large hematomas, mucosal bleeding and epistaxis - typical signs of primary ITP and had very low platelet counts. Additionally, we compared these results in ITP to those from healthy children and children with cTP.

In plasma from primary ITP patients we demonstrated the presence of elevated cytokines/chemokines of the Th1 as well as Th2 commitment (Figure 2) that is illustrated in a model in Figure 5. We showed that patients with acute ITP have increased levels of GM-CSF suggesting that GM-CSF is upregulated in ITP by macrophages in response to the low platelet count. These findings were in support with the report by Abboud et al. [10] (Figure 2C) who discussed that GM-CSF stimulates the proliferation of megakaryocytes and might be a regulator of platelet production [10]. Additionally, we demonstrated that patients with acute ITP have increased plasma levels of the Th1 cell cytokines IFN-γ, TNF-β and IL-2 (Figure 2A) as well as of the Th2 cytokines IL-6, IL-10 and IL-13 that differentiate B cells into plasma cells (Figure 2B). Here we show that macrophages are activated in ITP patients as we observed increased levels of IFN-γ and IL-6. Also T cells are activated in ITP patients as indicated by increased levels of IL-10, IL-13, IL-6 and TNF-α. Higher concentrations of IL-6 well as of IFN-γ and TNF-α were already detected in earlier studies in chronic ITP.

In this study, we demonstrated that ITP patients have increased plasma levels of IFN-γ. It is known that cytotoxic T cells produce IFN-γ and thereby induce an activation of macrophages. Thus we assume that an increase in IFN-γ is responsible for the in ITP patients observed activation of macrophages. Olsson et al. reported that ITP is associated with a T cell mediated cytotoxicity towards platelets [21]. They observed increased cytotoxic genes such as Apo-1/Fas, granzymes and perforin as well as the Th1 response cytokines IFN-γ and IL-2 in CD3+ cells from active ITP patients [21]. We suggest that this observed cytotoxicity leads to caspase activation in the ITP patient's platelets that we have observed in a recent study [19]. However, as shown in Table 2 we were not able to find any association of elevated plasma cytokine levels to an increased proportion of platelets with activated caspases in ITP patients. We therefore can not conclude that an increase in cytokines leads to an activation of caspases and cytotoxic T cells as well as granzymes and perforin have to be investigated in acute ITP patients.

In contrast to our previous study where platelet apoptotic events were ameliorated by IVIg treatment [19], we did not find a significant decrease of plasma cytokine levels after IVIg treatment although cytokines have a short half-life. This finding assumes that IVIg does not act on cytokine receptors. In this study we demonstrated that ITP patients at diagnosis have increased levels of TNF-α. In mice, TNF-α has been reported to induce thrombocytopenia and activation of caspase-3 [25]. However, while activation of caspase-3 was decreased after treatment with IVIg, TNF-α levels were not. We speculate that IVIg might block the cytokine receptors but not the cytokine production itself so that we observe a reduction in activated caspase-3 but not in cytokine levels. As TNF-α did not correlate to caspase activation we suggest that TNF-α is not responsible for triggering apoptosis in ITP patients. Furthermore, we did not observe increased levels of BAFF in acute ITP patients (Figure 4). In an earlier study of chronic ITP, platelet apoptosis was shown to correlate with increased BAFF concentrations [20]. In vitro assays presented that recombinant human BAFF increased platelet apoptosis and levels of IFN-γ [20]. However, while we detected increased IFN-γ in our study in plasma of acute ITP patients we did not detect significant altered BAFF concentrations and might consider including more subjects.

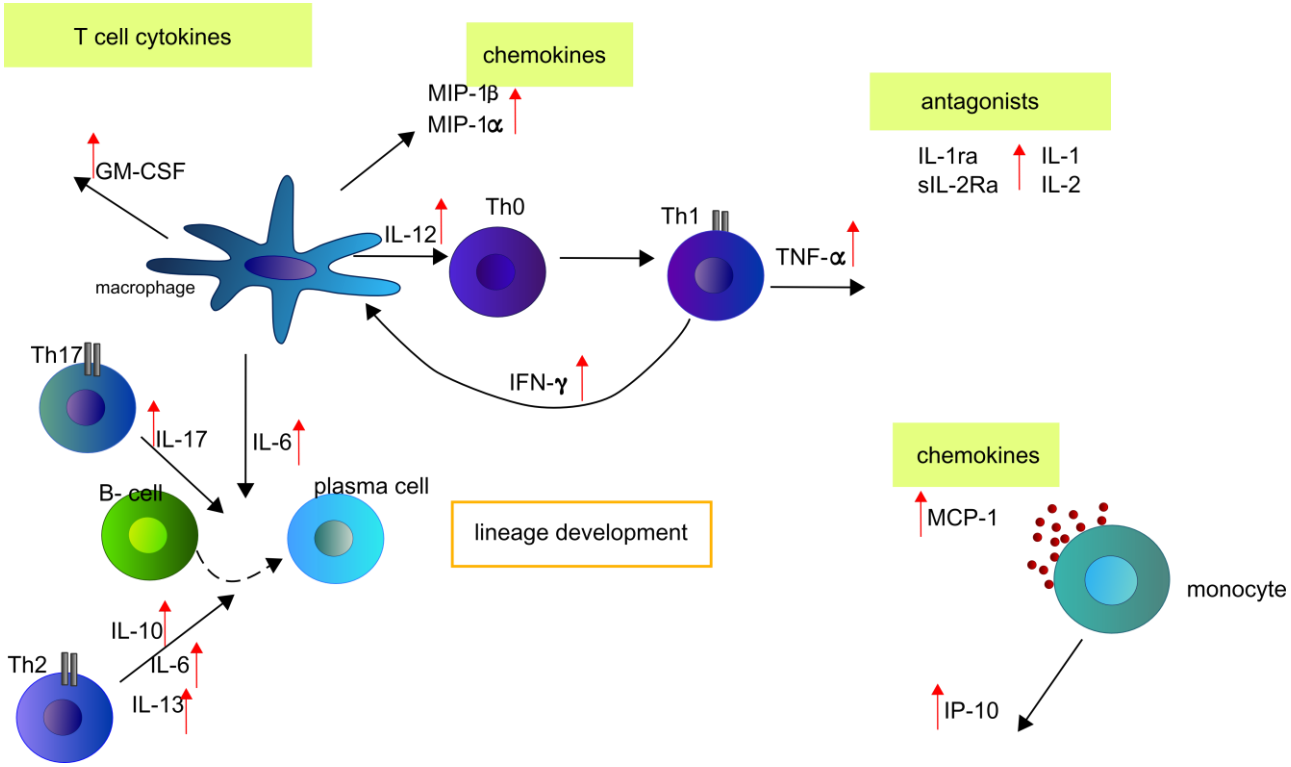

Figure 5. Increased plasma levels of cytokines involved in patients with acute ITP. ITP patients at diagnosis show increased plasma levels of cytokines that are involved in lineage development. The Th2 cytokines IL-6, IL10 and IL-13 as well as IL17 differentiate B cells into plasma cells. IL-12 differentiates Th cells into Th1 cells via activated macrophages. Th1 cells induce a production of TNFα and IFN-γ. Furthermore, activated macrophages also produce IL-6 and GM-CSF, cytokines that are also increased in ITP patients as well as the chemokines MIP-1α and MIP-1β. ITP patients at diagnosis further presented increased levels of the chemokines MCP-1 and IP-10. We also detected elevated plasma levels of IL-1 and the agonists IL-1ra and sIL-2Ra in ITP patients.

Furthermore, in plasma of ITP patients we detected increased levels of the receptor agonists IL-1ra and sIL-2Ra that function as scavengers (Figure 3A). IL-1ra acts in response to IL-1 and sIL-2Ra in response to IL-2. We also found elevated plasma levels of IL-1 (Figure 3A) as well as of IL-2 (Figure 2A). We also detected increased plasma levels of IP-10 (Figure 3B) confirming that IP-10 is secreted in response to elevated IFN-γ levels (Figure 2A). We also detected increased levels of the chemokines MIP-1α, MIP-1β as well as MCP-1 in plasma of ITP patients. This finding confirms that in ITP patients macrophages and monocytes are activated.

We found that acute ITP patients have increased plasma levels of Th17, however, they were not statistically significant from control (Figure 2D). IL-17 mediates B cell differentiation as do IL-6, IL-10 and IL-13 (Figure 5) [9]. Th17 have been shown to be involved in autoimmune diseases [7] [22] and recently to have a role in chronic ITP [6] [9]. Recently it was also reported that patients with untreated active ITP have increased levels of IL-27, a cytokine of the IL-6/IL-12 family [23]. IL-27 can regulate T cell differentiation and in ITP IL-27 might be responsible for induction of Th1 cells and pro-inflammatory cytokines as TNF-α and IFN-γ [23]. IL-27 was not included in our measurements, but as IL-27 has a similar function as IL-6 or IL-12 that are increased in our study population its concentration in acute ITP might also be higher. Furthermore, TNF-α and IL-6 have been shown to induce an upregulation of the tissue factor that mediates the initiation of the coagulation process [24]. This might be a negative feedback in response due to the hemostatic abnormality, low platelet and bleeding in ITP patients. Unfortunately, TGF-β, that has also been associated with chronic ITP in several studies, was neither included in our cytokine panel.

Plasma concentrations of IL-8 and MCP-1 were increased in both cTP and in ITP patients compared to the control group suggesting that the increase occurs in response to the thrombocytopenia. Cytokine levels statistically increased in ITP but not in cTP were TNFα, IL-10, IL-13, IPL-12 p40, IL-1ra, IL-1a, MIP-1α and MIP-1β as well as MDC indicating that they might be induced by a thrombocytopenia but rather by an autoimmune disease. In contrast to ITP, in a thrombocytopenia due to chemotherapy, proliferation of cells is probably impaired. Chemotherapy interferes with growth and proliferation of cells and megakaryocytes die due to cytotoxicity of the chemotherapy. Due to a defective megakaryopoiesis in the bone marrow, platelets are not produced anymore resulting in a low platelet count. In cTP we did not observe any activated caspases (described in the study of Chapter 2) because no new platelets are produced in response to chemotherapy. Here we detected that cTP patients have increased plasma levels of hematopoietic growth factors such as G-CSF, Flt-3 ligand, EGF or FGF-2 as well as the vascular endothelial growth factor VEGF, molecules that are involved in the progression of tumors.

As most studies concerning cytokines included chronic ITP patients and as we received different results, we conclude that alterations of concentrations of specific cytokines may be involved in the chronic history. In agreement with previous observations in chronic ITP [4] [5] [9], we also observed an upregulation of the Th1 lineage in acute ITP. We were not able to correlate the observed increase in plasma cytokine levels to infections prior or at diagnosis as patients with no infection also presented increased cytokine levels (Table 2). However, we cannot exclude that chronic ongoing inflammation processes are responsible for the induction of cytokines in ITP patients.

To summarize, our study demonstrated that acute ITP patients have increased plasma cytokine levels of TNF-α, IFN-γ, IL-6, IL-10 and IL-13 at initial presentation suggesting that these cytokines contribute to the pathogenesis of the disease. Therefore, these cytokines might be promising targets for new therapy approaches. According to our findings we conclude that ITP patients at diagnosis have activated macrophages, B cells and T cells (Figure 5) consistent with the ITP model where macrophages induce the stimulation of B and T cells and remove platelets from the system by binding to the anti-platelet autoantibodies [1].

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Authorship and Disclosures

JW, OS and SK designed the study; JW: performed all experiments except the experiment of Figure 4 (performed by OS); JW, OS and JP: analyzed data; JW, JP and OS: wrote the manuscript. The authors have no conflicts of interest to declare.

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CHAPTER 6

General discussion

Despite the finding that anuclear platelets possess pro- and anti-apoptotic proteins and undergo apoptotic events [1], their apoptotic signaling pathway is not entirely understood. Proteins as Omi/HtrA2 or XIAP of the intrinsic pathway have not been investigated yet in platelets. In recent findings, apoptotic changes were discussed in a mouse model of the platelet disorder immune thrombocytopenia (ITP) [2] [3].

Pediatric ITP is a common and benign platelet autoimmune disorder that presents as a sudden onset of petechiae and a very low platelet count in formerly healthy children, usually after a 1 to 3 weeks preceding viral infection. Although ITP is well characterized, the pathophysiology leading to the platelet consumption is not entirely understood [4] [5] nor why most patients recover from ITP within months and others develop into a chronic ITP. The current working model of ITP suggests that autoantibodies targeting platelet surface glycoproteins are responsible for an enhanced Fc receptor mediated platelet clearance.

One goal of the present work was to characterize platelets in ITP in regard to platelet apoptosis and platelet function. Another aim was to identify apoptotic proteins in health and understand the possible apoptotic signaling pathways. A further aim was to identify a possible trigger for apoptosis in ITP patients.

In a prognostic pediatric ITP study, described in Chapter 2, we demonstrated that ITP patients at diagnosis have increased apoptotic events in platelets as activated caspases, increased PS exposure and microparticles [6]. These apoptotic manifestations in platelets were ameliorated by IVIg treatment. We also found that platelets of ITP patients have a reduced $\Delta\Psi m$. However, the reduced $\Delta\Psi m$ was not ameliorated by IVIg suggesting that IVIg, as observed in the ITP mouse model, only inhibits downstream apoptotic events in platelets [2]. We also detected a slight increase in caspase activation in platelets of chronic ITP (Chapter 3). However, this observed increase was much lower than in acute ITP and is based on preliminary data, but suggests that in chronic ITP the pathophysiology eventually could be mediated by different factors than in acute ITP.

In the study described in Chapter 3 we found that platelets of ITP patients at diagnosis have increased pre-activation and reduced thrombin activation. We detected an increased surface expression of CD63 and CD62P compared to control, confirming the secretion of lysosomal or granule proteins in acute ITP patients, an early step in platelet activation [7]. In contrast,

we did not observe an increased PAC-1 binding indicating that there was no conformational change in GPIIb/IIIa and therefore the fibrinogen binding site was not exposed [8]. As there was no active GPIIb/IIIa detected we conclude that platelets are probably not fully pre-activated in ITP patients. We speculate that in response to the low platelet count and due to the expressed CD62P, ITP platelets increase their adhesiveness. Although CD63 and CD62P surface expression in circulating ITP platelets was higher, the capacity for further activation of these platelets by thrombin stimulation was impaired. Thrombin-induced stimulation was also lower in cTP patients but in contrast to ITP, CD63 and CD62P surface expression was not increased in cTP patients. Furthermore, in the study of Chapter 3 we described that ITP patients have a reduced endogenous thrombin potential. IVIg improved both ETP and thrombin activation of ITP platelets as well as the bleeding symptoms. The low ETP observed in ITP patients at diagnosis as well as in chronic ITP might be responsible for the bleeding observed in ITP. ETP correlated to the platelet count thus might be a biological marker for estimating the bleeding risk in ITP patients. However, cTP patients also presented a reduced platelet count but had an ETP comparable to healthy controls. Furthermore, it was assessed that the bleeding severity can not be predicted by thrombin generation [9] [10]. ETP did not correlate with CD63 and CD62P expression verifying that an increased expression of these proteins is not responsible for the low ETP.

We also found that ITP patients at diagnosis have increased reticulated platelets that are defined as the young circulating platelets as well as an increased platelet size (Chapter 2). With these data we speculate that CD62P expression differs in younger, larger circulating platelets from normal-sized platelets.

We did not find a correlation of CD63 and CD62P expression with activation of caspase-3 indicating that pre-activated platelets (Chapter 2 and 3) are not responsible for an induction of caspase-3. This confirms the finding that platelet activation and platelet apoptosis are different events that occur sequentially [11] [12] [13]. The increase in PS exposure as well as microparticles might contribute to expression of CD63 and CD62P that we observed in platelets of ITP patients leading to an enhanced coagulation. However, increased PS exposure as well as elevated microparticles are also considered as apoptotic manifestations. A small proportion of platelets presented PS exposure as well as caspase activation indicating that this proportion was clearly involved in platelet apoptosis [6] (Chapter 2). Thrombin- as well as A23187- stimulation of resting platelets lead to an increase in caspase activation in contrast to the thrombin-reduced activation of CD63 and CD62P (Chapter 3). This suggests that the action of thrombin on platelets is not totally impaired in ITP.

It is not established what initiates apoptosis in ITP, whether the intrinsic pathway is triggered due to a disruption of the Ca^{2+} homeostasis or by the extrinsic pathway via a death receptor. At diagnosis of ITP, we observe signs of the intrinsic apoptotic pathway: a decrease in $\Delta\Psi m$, an activation of caspase-9 as well as of its effector caspase-3 in the patient's platelets (Chapter 2). However, it remains to be elucidated whether cytochrome c is released in platelets of ITP patients. We also found an activation of caspase-8 in platelets of ITP patients that in nucleated cells is part of the extrinsic apoptotic pathway. We speculate that also Bid signaling might be involved in platelet apoptosis of pediatric ITP. In nucleated cells, Bid connects the extrinsic to the intrinsic pathway while activated caspase-8 processes Bid into truncated Bid that gets localized to the mitochondria [14]. Cleavage of procaspase-8 is mediated by death receptor signaling. However, in platelets apart from DcR2 no death receptors were identified so far [15]. We were unable to detect any receptors in platelets as TRAIL, DR3 or DR5. However, we found the death domain FADD that links death receptors to caspase-8 activation (Supplement of Chapter 4). We also confirmed the expression of Bid in platelets (Supplement of Chapter 4). The presence of FADD and Bid, and the finding that caspase-8 is activated by physiological and artificial compounds in platelets of healthy subjects as well as of ITP, indicate that platelets contain the extrinsic apoptotic pathway.

Platelets have most important players of the apoptotic signaling pathway such as caspases, Apaf-1, cytochrome c, the anti-apoptotic protein Bcl-XL or the pro-apoptotic protein Bax [1] [12] [16] [17] [15]. However, the entire signaling pathway has not been described yet, especially proteins as XIAP or Omi/HtrA2 have not been analyzed. We identified two factors of the intrinsic apoptotic signaling pathway in human platelets- the pro-apoptotic factor Omi/HtrA2 as well as its target inhibitor XIAP, an anti-apoptotic protein (Chapter 4). We demonstrated that platelets release Omi/HtrA2 from mitochondria into the cytosol upon onset of apoptosis. We also confirmed that Smac/Diablo and cytochrome c are released from mitochondria into the cytosol as was shown previously [15] [13]. By using an inhibitor of Omi/HtrA2, Ucf-101 [18], we showed that apoptotic events were reduced and by using an inhibitor of XIAP, embelin [19], we demonstrated that apoptotic events were increased. Thus, we verified that as in nucleated cells Omi/HtrA2 is a pro-apoptotic protein and that caspase-3 and 9 are inhibited by XIAP. We further indicated that the inhibition of Omi/HtrA2 by Ucf-101 occurs in the cytosol, therefore after $\Delta\Psi m$ depolarization.

In mice, TNF-α, a ligand for the receptor TNFR1 or TNFR2 in nucleated cells, has been reported to induce thrombocytopenia and activation of caspase-3 in healthy platelets [20]. In pediatric ITP, it is discussed that apart from macrophages also B and T cells are involved in the pathophysiology [5]. Furthermore in ITP, activated cytotoxic T cells were found that

induced platelet lysis and elevated expression of FasL and TNF-α in these cytotoxic T cells was shown to be present [21] [22]. To investigate whether an extrinsic trigger as a ligand for death receptors such as TNF-α may be responsible for induction of apoptosis in platelets of children with ITP, we studied cytokines in plasma of ITP patients (Chapter 5). We found that ITP patients at diagnosis have increased plasma levels of the Th1 cell cytokines IFN-γ, TNF-α and IL-2 as well as of the Th2 cytokines IL-6, IL-10 and IL13 and increased GM-SCF (Chapter 4). However, while activation of caspase-3 was decreased after treatment with IVIg, TNF-α levels were not. Neither did we found a correlation of caspase activation with TNF-α nor with other cytokines that showed increased levels at diagnosis. Thus, it is unlikely that an increased TNF-α concentration is responsible for triggering apoptosis. However, other cytokines not included in this study, might still be candidates for inducing apoptosis. Or, elevated cytokines might be involved in chronic ITP. We consider analyzing cytokines in chronic ITP patients also as cytokines are involved in immune-mediated inflammations. In platelets of patients with chronic ITP we detected less activated caspases suggesting that other factors are involved in the pathophysiology of chronic ITP than of acute ITP.

The present work generated findings of activated caspases in platelets in acute pediatric ITP patients. We found that in ITP patients, after an increase in platelet counts by IVIg, platelet apoptotic and activation events as well as the low endogenous thrombin potential were ameliorated. We therefore prove the efficacy of the IVIg treatment in ITP patients. However, we do not know how IVIg decreases caspase activity in platelets of ITP. In a next step, we will administer anti-GPIIb antibodies as well as IVIg in healthy platelets in vitro in order to investigate whether anti-GPIIb antibodies induce caspase activity and whether IVIg reverses this activity. Of great note is to find out which apoptotic signaling is activated in ITP platelets and whether platelets have death receptors. For this, it would be important to detect more apoptotic changes in ITP platelets such as a release of cytochrome c or Omi/HtrA2. However, due to the low platelet count of ITP patients at diagnosis it is nearly impossible to detect proteins by Western blot and we might include other approaches in the future such as intracellular flow cytometry staining. At the moment we can not show that ETP or any other laboratory parameter correlates to bleeding signs. It will be our continued intention to estimate by a biological marker if a patient is at a high bleeding risk such as cerebral bleeding or not.

To summarize, we showed that ITP patients at diagnosis have increased manifestations of platelet apoptosis, decreased thrombin-induced platelet stimulation as well as a reduced ETP. We proved that after IVIg treatment all apoptotic manifestations except $\Delta\Psi m$ were normalized. Also as the decreased thrombin-induced platelet stimulation and the reduced

ETP were improved. Additionally we verified that platelets contain the important apoptotic proteins to have a functional apoptotic signaling. We assume that an apoptotic-like signaling is present in ITP platelets at initial presentation.

The significance of this study is to gain an increase in the understanding of the pathophysiology of ITP by analyses of apoptotic signaling and thrombin generation in platelets of ITP patients. By extending our findings to a larger study it might be possible to find a clear correlation between clinical parameters and laboratory data. In future studies, it will be important to distinguish which ITP patient develops into low risk or high risk of bleeding or into a chronic ITP. A clinical aim of this study is not to treat ITP children in Switzerland with low risk of bleeding and spontaneous remission with IVIg, an expensive and invasive treatment.

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Abbreviations

BAFF	B cell activating factor
BH3	Bcl-2 homology domain 3
BIR	Baculovirus inhibitor of apoptosis repeats
BSS	Bernard-Soulier syndrome
CAD	Caspase-activated DNase
Ca2+	Calcium
CaM	Calmodulin
CARD	Caspase recruitment domain
CsA	Cyclosporin A
C+T	Collagen and thrombin
cTP	Chronic ITP
CypD	Cyclophilin D
DTS	Dense tubular system
$\Delta\psi m$	Mitochondrial membrane potential
ER	Endoplasmatic Reticulum
ETP	Endogenous thrombin potential
Fc receptor	Fragment crystallizable receptor
FLICA	Fluorochrome inhibitors of caspases
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	Glycoprotein
Hsp	Heat shock protein
IAP	Inhibitor of apoptotic proteins
IL	Interleukin
IMS	Mitochondrial intermembrane space
IP	Immunoprecipitation
ITP	Immune thrombocytopenia
IVIg	Intravenous immunoglobuline
MFI	Geometric mean fluorescence intensity
MOMP	Mitochondrial outer membrane permeabilization
MPs	Platelet-derived microparticles
MPTP	Mitochondrial permeability transition pore
PCs	Platelet concentrates
RES	Reticuloendothelial system
PKC	Protein kinase C

PPP	Platelet poor plasma
PRP	Platelet rich plasma
PRT	Pathogen reduction technology
PS	Phosphatidylserine
RT	Room temperature
TAH	Tyrodes Hepes buffer with albumin
TF	Tissue factor
TGF-β1	Transforming growth factor β-1
Th	T helper cells
TH	Tyrodes Hepes buffer
TNF	Tumor necrosis factor
TMEM	Transmembrane protein
TMRE	Tetramethylrhodamine-ethyl-ester
TPO	Thrombopoietin
vWF	von Willebrand Factor

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Curriculum vitae

Jeannine WINKLER

Date of birth	13.08.1982
Home town	Winterthur, ZH
Nationality	Swiss
Marital status	Single

EDUCATION

05/2009 – 07/2012	**PhD studies in Biology – „*Platelet apoptosis in health and disease"*** Children's Hospital Zurich – Life Science Zurich Graduate School – University of Zurich, Switzerland
02/2006 – 04/2007	**Master of Science in Molecular Biology – *„AgGic1 and AgBem4, two proteins involved in polarity establishment"*** Biozentrum University of Basel – Switzerland
10/2002 – 07/2005	**Bachelor of Science in Biology** Biozentrum University of Basel – Switzerland
08/1998 – 12/2001	**Matura Type B** (Latin), Gymnasium Muttenz

GRANTS

2009	**Donation UBS AG by order of a client**

RESEARCH EXPERIENCE

05/2009 – 07/2012 **Children's Hospital Zurich, University of Zurich,** Switzerland

- Identification of apoptotic proteins in human platelets by Western blot and Immunoprecipitation
- Induction and prevention of platelet apoptosis with agonists or specific inhibitors of pro- and anti-apoptotic proteins
- Analysis of apoptotic events and platelet activation in platelets from patients with pediatric ITP patients by Flow cytometry
- Analysis of cytokines in ITP patients by Luminex Technology

04/2008 – 03/2009 **Actelion Pharmaceuticals Ltd, Allschwil,** Switzerland

- Cloning and sequencing of plasmids
- Gene expression analysis via quantitative PCR
- Performance of microarrays

02/2006 – 08/2007 **Biozentrum University of Basel, Switzerland**
- Analysis of polar growth in *A.Gossypii* with genomic-, cell-biologic- and computer-based methods
- Gene-deletions and -fusions
- Videomicroscopy of growth in *A. Gossypii wild type and deletion phenotypes*

08/2005 – 10/2005 **Technical University of Loja (UTPL), Loja,** Ecuador
- Toxicity and activity of medical plants
- Analysis of mycorrhizae in roots of orchids

PUBLICATIONS

Winkler J, Kroiss S, Rand ML, Azzouzi I, Bang KWA, Speer O, Schmugge M. *Platelet apoptosis in paediatric immune thrombocytopenia is ameliorated by intravenous immunoglobulin.* British Journal of Haematology, 2012. **156**(4): p. 508–515.

Azzouzi I, Moest H, **Winkler J**, Fauchère J-C, Gerber AP, Wollscheid B, Stoffel M, Schmugge M, Speer O, *MicroRNA-96 Directly Inhibits γ-Globin Expression in Human Erythropoiesis*. PLoS ONE, 2011. **6**(7): e22838.

CONFERENCE PRESENTATIONS

„Platelet apoptosis in paediatric immune thrombocytopenia is ameliorated by intravenous immunoglobulin.“ Young Researcher Day, Luzern, Switzerland, May 2012. Talk.

“Activated caspases-3, -8 and -9 occur in platelets from children with immune, but not chemotherapy-related, thrombocytopenia“ FZK (children’s research center) retreat, Wädenswil, Switzerland, November 2011. Poster

„Activation of caspases-8 and -9 occurs in platelets from children with immune, but not chemotherapy-related, thrombocytopenia.“ American society of hematology annual meeting (ASH), Orlando, USA, December. 2010. Poster and Talk

“Platelet apoptosis in childhood immune thrombocytopenia (ITP)”. SPOG Scientific Meeting, Lugano, Switzerland, January 2010. Talk

LANGUAGES

German	native language
English	fluent
Spanish	fluent
French	basic knowledge